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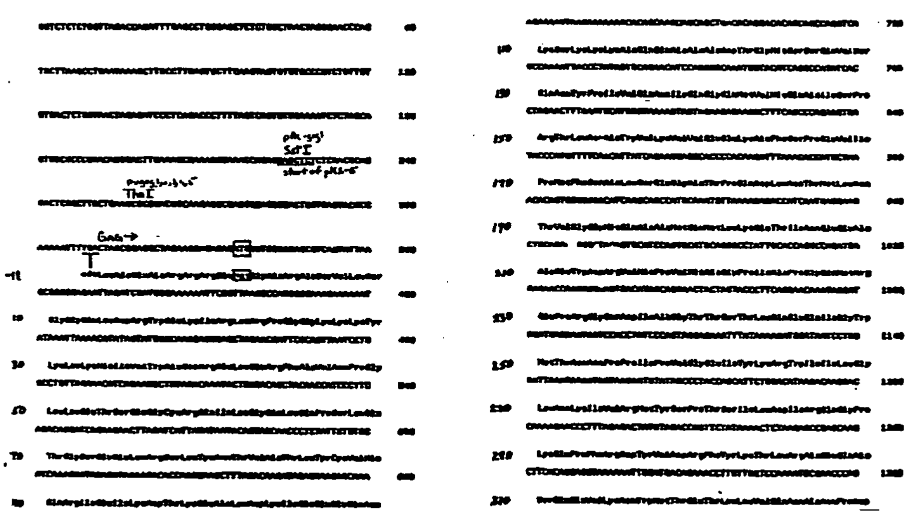

(57) Abstract

Recombinant viruses which direct the expression of peptides or proteins related to epitopes of LAV/HTLV III, the etiological agent of Lymphadenopathy Syndrome and Acquired Immune Deficiency Syndrome. These viruses can be formulated as viral vaccines or in multivalent vaccines that protect against LAV/HTLV III infection. Antigenic peptides and proteins related to epitopes of LAV/HTLV III are also described. These can be prepared using recombinant DNA techniques or by chemical synthesis and can be used as immunogens in subunit vaccines or as antigens in diagnostic immunoassays.

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VACCINES AND IMMUNOASSAYS FOR ACQUIRED IMMUNE DEFICIENCY SYNDROME

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1. FIELD OF THE INVENTION

The present invention is directed to viruses that express peptides and proteins related to epitopes of Lymphadenopathy Associated Virus (LAV/HTLV III)/Human T-Cell Leukemia Virus (HTLV-III), the etiological agent of Lymphadenopathy Syndrome (LAS) and Acquired Immune Deficiency Syndrome (AIDS). The viruses of the present invention which express LAV/HTLV III related peptides that induce a protective immune response may be used as immunogens in viral vaccine formulations for LAS or AIDS or in multivalent vaccine formulations. In fact, infectious viruses of the present invention which can multiply in a host without causing disease may be used in live viral vaccine formulations that provide a prolonged immunogenic stimulus and give rise to substantial immunity.

The present invention is also directed to peptides and proteins related to epitopes of LAV/HTLV III that may be used as immunogens in subunit vaccine formulations for LAS or AIDS, or in multivalent vaccine formulations, or as antigens in diagnostic immunoassays for LAS or AIDS. These peptides and proteins may be produced using recombinant DNA techniques in any vector-host system or they may be synthesized by chemical methods. Accordingly, the invention is also directed to the construction of novel DNA sequences and vectors including plasmid DNA, and viral DNA such as human viruses, animal viruses, insect viruses or bacteriophages which can be used to direct the expression of LAV/HTLV III related peptides and proteins in appropriate host cells from which the peptides and proteins may be purified. Chemical methods for the synthesis of LAV/HTLV III related peptides and proteins are also described.

In a specific embodiment of the present invention, recombinant vaccinia viruses were used to produce LAV/HTLV III envelope or core related proteins. To this end, DNA sequences of env or gag, which encode the envelope

glycoproteins or core structural proteins of LAV/HTLV III, respectively, were inserted into vaccinia vectors which are capable of directing the expression of the LAV/HTLV III genes in an appropriate host. The LAV/HTLV III envelope related proteins produced by the recombinant vaccinia viruses were found to be antigenic and immunogenic and capable of eliciting humoral and cell mediated immunity in sub-human primates. The LAV/HTLV III gag related proteins produced by the recombinant vaccinia viruses were immunoreactive proteins that contained the major epitopes of the authentic core proteins.

The recombinant vaccinia viruses which express the LAV/HTLV III envelope related proteins may be used alone or in conjunction with the vaccinia recombinants which express other LAV/HTLV III related proteins (such as core structural proteins) in viral vaccine formulations. Alternatively, the LAV/HTLV III related proteins produced by the recombinant viruses can be purified or chemically synthesized and used as immunogens in subunit vaccine formulations. Since the LAV/HTLV III related protein(s) will be recognized as "foreign" in the host animal, a humoral and/or cell mediated immune response should be raised against the protein or combination of proteins. In a properly prepared vaccine formulation, this should protect the host against subsequent LAV/HTLV III infections.

In another specific embodiment of the present invention, recombinant baculoviruses (Autographa californica nuclear polyhedrosis virus or AcNPV) were used to produce LAV/HTLV III envelope and core related proteins. To this end, DNA sequences of env or gag, which encode envelope or core structural proteins, respectively, were inserted into baculovirus vectors which are capable of directing the expression of the LAV/HTLV III genes in an appropriate host. The LAV/HTLV III proteins produced by the recombinant

baculoviruses were found to be antigenic as measured by radioimmunoprecipitation and ELISA.

This invention also provides for the production of LAV/HTLV III antigens which are of general importance in human medicine. These include the use of the peptides and proteins of the present invention as reagents in immunoassays such as ELISA tests and radioimmunoassays which are useful as diagnostic tools for the detection of antibodies specific for LAV/HTLV III in blood samples, body fluids, tissues, etc. In addition, these reagents will be a valuable tool in elucidating the mechanism of pathogenesis of LAV/HTLV III.

2. BACKGROUND OF THE INVENTION

2.1. AIDS VIRUS

Acquired Immunodeficiency Syndrome (AIDS) is a disease characterized by severe immune deficiency due primarily to impairment of the patient's cell mediated immune response (Gottlieb, M. et al., 1981, N. Engl. J. Med. 305:1425; Masur, J. et al., 1981, N. Engl. J. Med. 305:1431). Two clinical presentations of the disease are recognized: (a) a prodromal phase called Lymphadenopathy Syndrome (LAS) characterized by chronic lymphadenopathy, leukopenia and a quantitative decrease in peripheral blood helper cells (OKT4 cells) leading to a reversal of the normal peripheral helper to suppressor T lymphocyte ratio (OKT4:OKT8) which shifts from 2 to 0.1 or less as the disease progresses; and (b) an immunodeficient state characterized by a decrease in OKT4 cells and reversal of the normal OKT4:OKT8 ratio, absolute lymphopenia, and repetitive opportunistic infections mainly by Pneumocystis carnii; this latter phase is ultimately associated with death in the majority of cases. Certain subsets of patients have increased incidence of lymphoma and Kaposi's sarcoma. Currently, there is no cure or therapy for the disease.

Epidemiological data along with information concerning the types of patients that acquired the disease suggested that an infectious agent transmitted by intimate contact might be the cause of the disease. Subsequently three groups have provided strong evidence that the causative agent of AIDS is a retrovirus with a tropism for helper T lymphocytes. These groups are:

(a) R.C. Gallo and coworkers at the National Institute of Health were able to isolate a cytopathic retrovirus (HTLV III) from patients with AIDS and pre-AIDS (Gallo, R.C. et.al., 1984, Science 224:500; Popovic, M. et al., 1984, Science 224:497). They also detected antibodies against HTLV III in the serum of patients with AIDS.

(b) L. Montagnier and coworkers at the Pasteur Institute isolated a T-lymphotropic retrovirus (LAV/HTLV III) from a patient who presented with cervical lymphadenopathy and was at risk for AIDS (Barre-Sinoussi, F., et al., 1983, Science 220:868). This group was also able to demonstrate antibodies against LAV/HTLV III in serum from AIDS patients (Kalyansraman, V.S., et al., 1984, Science 225: 321). Moreover, they were able to isolate LAV/HTLV III from the lymphocytes of a patient who developed AIDS after receiving blood from a donor who developed AIDS (Feorino, P.M., et al., 1984, Science 225:69).

(c) J. Levy and coworkers isolated infectious retroviruses (termed AIDS-associated retrovirus, or ARV) from the peripheral mononuclear cells of patients with AIDS (Levy, J.A., et al., 1984, Science 225:840).

Although all three viruses were isolated independently, they all probably belong to the same retrovirus subgroup

(Levy, J.A., et al., 1984, Science 225:840) and will be collectively referred to herein as LAV/HTLV III.

The general structure of retroviruses is that of a ribonucleoprotein core surrounded by a lipid containing envelope which the virus acquires during the course of cell budding. Embedded within the envelope and projecting outward are the viral encoded glycoproteins. These determine the host range of the virus and react with specific receptors on the surface of susceptible cells. Neutralizing antibodies are thought to bind to envelope glycoproteins and block their interaction with receptors on the surface of cells (pp. 534-535 in, The Molecular Biology of Tumor Viruses, ed. J. Tooze, 1973, Cold Spring Harbor Laboratory; pp. 226-227 and 236-237 in, RNA Tumor Viruses, ed. R. Weiss, N. Teich, H. Varmus, and J. Coffin, 1982, Cold Spring Harbor Laboratory.). In the specific case of LAV/HTLV III, there is evidence that the T_4 antigen, present on a subset of T lymphocytes, is the receptor or a component of the receptor for the virus (Dalgleish, A.G., et al., 1984, Nature 312:763; Klatzmann, D., et al., 1984, Nature 312:767).

The RNA genome of LAV/HTLV III is diagrammed in FIG. 1. Three genes are generally recognized: the gag gene codes for the internal structural proteins (core proteins) of the virus and defines the viral group-specific antigens. The pol gene codes for the virion associated reverse transcriptase. The env gene codes for the viral glycoproteins. Other regions marked sor and 3'-orf denote areas of the genome containing open reading frames; the function of these regions is not known at present.

2.2. VACCINES

A number of methods are currently employed for the prevention and treatment of viral infections. These include the use of vaccines which elicit an active immune response,

treatment with chemotherapeutic agents and interferon treatment.

Traditional ways of preparing vaccines include the use of inactivated or attenuated viruses. Inactivation of the virus renders it harmless as a biological agent but does not destroy its immunogenicity. Injection of these "killed" virus particles into a host will then elicit an immune response capable of neutralizing a future infection with a live virus. However, a major concern in the use of killed vaccines (using inactivated virus) is failure to inactivate all the virus particles. Even when this is accomplished, since killed viruses do not multiply in their host, the immunity achieved is often short-lived and additional immunizations are usually required. Finally, the inactivation process may alter the viral proteins rendering them less effective as immunogens.

Attenuation refers to the production of virus strains which have essentially lost their disease producing ability. One way to accomplish this is to subject the virus to unusual growth conditions and/or frequent passage in cell culture. Viral mutants are then selected which have lost virulence, yet are capable of eliciting an immune response. The attenuated viruses generally make good immunogens since they actually replicate in the host cell and elicit long-lasting immunity. However, several problems are encountered with the use of live vaccines, the most worrisome being insufficient attenuation.

An alternative to the above methods is the use of subunit vaccines. This method involves immunization only with those proteins which contain the relevant immunological material. For many enveloped viruses, the virally encoded glycoprotein contains those epitopes which are capable of eliciting neutralizing antibodies; these include the glycoproteins of La Crosse Virus (Gonzalez-Scarano, F., Shope, R.E., Calisher, C.E. and Nathanson, N., 1982, Virology

120:42.), Neonatal Calf Diarrhea Virus (Matsuno, S. and Inouye, S., 1983, Infection and Immunity 39: 155), Venezuelan Equine Encephalomyelitis Virus (Mathews, J.H. and Roehrig, J.T., 1982, J. Imm. 129:2763), Punta Toro Virus (Dalrymple, J.M., Peters, C.J., Smith, J.F. and Gentry M.K., 1981, In "Replication of Negative Strand Viruses", D.H.L. Bishop and R.W. Compans, eds., p. 167. Elsevier, New York), Murine Leukemia Virus (Steeves, R.A., Strand, M. and August, J.T., 1974, J. Virol. 14:187), and Mouse Mammary Tumor Virus (Massey, R.J. and Schochetman, G., 1981, Virology 115:20). One advantage of subunit vaccines is that the irrelevant viral material is excluded.

Vaccines are often administered in conjunction with various adjuvants. The adjuvants aid in attaining a more durable and higher level of immunity, using smaller amounts of antigen in fewer doses than if the immunogen were administered alone. The mechanism of adjuvant action is complex and not completely understood. However, it may involve the stimulation of phagocytosis and other activities of the reticuloendothelial system as well as a delayed release and degradation of the antigen. Examples of adjuvants include Freund's adjuvant (complete or incomplete), Adjuvant 65 (containing peanut oil, mannide monooleate and aluminum monostearate), the pluronic polyol L-121, Avridine, and mineral gels such as aluminum hydroxide, aluminum phosphate, or alum. Freund's adjuvant is no longer used in vaccine formulations for humans because it contains nonmetabolizable mineral oil and is a potential carcinogen.

2.2.1. RECOMBINANT DNA TECHNIQUES AND VACCINIA VIRUS

The use of recombinant DNA technology for the production of subunit vaccines involves the molecular cloning and expression in an appropriate vector of the viral genetic information coding for those proteins which can elicit a neutralizing response in the host animal. All other genetic

information of the virus is excluded and only those proteins required to establish a neutralizing response are presented to the host animal. The host is never exposed to the whole virus and stands no risk of becoming infected.

Recently, an approach has been described which is potentially useful in the production of subunit vaccines (Mackett, M., Smith, G.L. and Moss, B., 1982, Proc. Natl. Acad. Sci. 79: 7415-7419; Mackett, M., Smith, G.L. and Moss, B., 1984, J. Virol. 49: 857-864; Panicali, D. and Paoletti, E., 1982, Proc. Nat'l. Acad. Sci. 79: 4927-4931). This approach involves the use of vaccinia virus as a vector to express foreign genes inserted into its genome. Upon introduction into host animals, the recombinant vaccinia virus expresses the inserted foreign gene and may thereby elicit a host immune response to such gene products. Since live recombinant vaccinia virus can be used as a vaccine, such an approach combines the advantages of both subunit and live vaccines.

Vaccinia virus contains a linear double-stranded DNA genome of approximately 187 kilobase pairs and replicates within the cytoplasm of infected cells. These viruses contain a complete transcriptional enzyme system (including capping, methylating and polyadenylating enzymes) within the virus core that is necessary for virus infectivity. Vaccinia virus transcriptional regulatory sequences (promoters) allow for initiation of transcription by vaccinia RNA polymerase but not by host cell RNA polymerase.

Expression of foreign DNA in recombinant vaccinia viruses requires the ligation of vaccinia promoters to protein coding DNA sequences of the foreign gene. Plasmid vectors, also called insertion vectors, have been constructed to insert chimeric genes into vaccinia virus. One type of insertion vector is composed of: (a) a vaccinia virus promoter including the transcriptional initiation site; (b) several unique restriction endonuclease cloning sites located

downstream from the transcriptional start site for insertion of foreign DNA fragments; (c) nonessential vaccinia virus DNA (such as the TK gene) flanking the promoter and cloning sites which direct insertion of the chimeric gene into the homologous nonessential region of the virus genome; and (d) a bacterial origin of replication and antibiotic resistance marker for replication and selection in E. coli. Examples of such vectors are described by MacKett (Mackett, M., Smith, G.L. and Moss, B., 1984, J. Virol. 49: 857-864).

Recombinant vaccinia viruses are produced by transfection of recombinant bacterial insertion plasmids containing the foreign gene into cells previously infected with vaccinia virus. Homologous recombination takes place within the infected cells and results in the insertion of the foreign gene into the viral genome. The infected cells can be screened using immunological techniques, DNA plaque hybridization, or genetic selection for recombinant viruses which subsequently can be isolated. These vaccinia recombinants retain their essential functions and infectivity and can be constructed to accommodate approximately 35 kilobases of foreign DNA.

Foreign gene expression can be detected by enzymatic or immunological assays (for example, immunoprecipitation, radioimmunoassay, or immunoblotting). Naturally occurring membrane glycoproteins produced from recombinant vaccinia infected cells are glycosylated and may be transported to the cell surface. High expression levels can be obtained by using strong promoters or by cloning multiple copies of a single gene.

2.2.2. RECOMBINANT DNA TECHNIQUES AND BACULOVIRUS

Recently, an approach has been described which is potentially useful for the production of recombinant proteins (Pennock et al., 1984, Mol. Cell Biol. 4:399; Smith et al., 1983, J. Virol. 46:584). This approach involves the use of a

baculovirus vector to express foreign genes inserted into its genome. Upon introduction into an appropriate host cell, the recombinant baculovirus expresses the foreign gene.

The prototype of the Baculoviridae family is <u>Autographa californica</u> nuclear polyhedrosis virus (AcNPV). The genome of AcNPV consists of a double stranded circular DNA species of 128 kilobase pairs which has been mapped with respect to several restriction sites (Smith, G.E. and Summers, M.D., 1978, Virology 89:517). The virus replicates in the nucleus of infected insect cells. Two forms of virus are produced as a result of wild type AcNPV infection of susceptible cells, occluded and non-occluded virus particles. The occluded virus particles are surrounded by a protein called polyhedra which is coded for by the polyhedrin gene (see Virol. 131:561-565, 1983). The occluded viral particles can be easily visualized in infected cells with the aid of a light microscope.

Expression of foreign DNA in recombinant baculoviruses requires the ligation of baculovirus promoters to protein coding DNA sequences of the foreign gene. Plasmid vectors, also called insertion vectors, have been constructed to insert chimeric genes into AcNPV. One type of insertion vector is composed of: (a) an AcNPV promoter including the transcriptional initiation site; (b) several unique restriction endonuclease cloning sites located downstream from the transcriptional start site for insertion of foreign DNA fragments; (c) nonessential AcNPV DNA (such as the polyhedrin gene) flanking the promoter and cloning sites which direct insertion of the chimeric gene into the homologous nonessential region of the virus genome; and (d) a bacterial origin of replication and antibiotic resistance marker for replication and selection in <u>E. coli</u>. Examples of such vectors are described by Miyamota et al. (1985, Mol. Cell. Biol. 5:2860).

Recombinant baculoviruses are produced by co-transfection of cells with recombinant bacterial insertion plasmids containing the gene, together with baculovirus DNA. Homologous recombination takes place within the infected cells and results in the insertion of the foreign gene into the viral genome. Once a foreign gene is inserted into the polyhedrin gene, occluded virus particles can no longer be produced and the resulting recombinant plaques can be screened visually for lack of occlusion bodies. The infected cells can also be screened using immunological techniques, DNA plaque hybridization, or genetic selection for recombinant viruses which subsequently can be isolated. These baculovirus recombinants retain their essential functions and infectivity.

Foreign gene expression can be detected by enzymatic or immunological assays (for example, immunoprecipitation, radioimmunoassay, or immunoblotting). High expression levels can be obtained by using strong promoters or by cloning multiple copies of a single gene.

3. SUMMARY OF THE INVENTION

Viruses which direct the expression of peptides or proteins related to epitopes of LAV/HTLV III are described. The recombinant viruses of the present invention which express LAV/HTLV III related epitopes that induce a protective immune response may be formulated as viral vaccines to protect humans against LAV/HTLV III infection. In a particular embodiment of the present invention, live virus vaccine formulations may be prepared using infectious viruses which express such LAV/HTLV III related epitopes in an infected host but do not cause disease in the host.

The invention is also directed to peptides or proteins related to the epitopes of LAV/HTLV III. Those which induce a protective immune response may be formulated in subunit vaccines or in multivalent vaccines to protect humans against

LAV/HTLV III infection. Alternatively, the peptides or proteins of the invention may be used in diagnostic assays for AIDS or LAS. The peptides or proteins of the present invention may be produced in and isolated from any host cell-expression vector system; these include, for example, animal or insect cell cultures infected with appropriate recombinant virus; microorganisms such as bacteria transfected with recombinant plasmids, cosmids or phages; and yeast transformed with recombinant plasmids. The present invention also provides methods, procedures and DNA constructions which are used for the expression of genetic information coding for the epitopes of LAV/HTLV III. Alternatively, the peptides and proteins of the present invention can be chemically synthesized.

In specific embodiments of the present invention detailed in the examples, gene sequences coding for the envelope glycoproteins or the core structural proteins of LAV/HTLV III (i.e., the LAV/HTLV III env or gag gene sequences, respectively) are inserted into plasmids so that an early vaccinia virus promoter is positioned 5′ to the initiation methionine sequence (ATG) of the LAV/HTLV III gene sequences, resulting in the construction of chimeric genes flanked by vaccinia thymidine kinase (TK) DNA sequences. These plasmids were transfected into cells which had been previously infected with wild-type vaccinia virus, thus allowing the chimeric LAV/HTLV III env or gag gene flanked by TK sequences to be recombined into the TK gene of the vaccinia virus. The cells were allowed to lyse and the resulting viruses were plaqued on TK^- cells. Recombinant viruses were selected by their ability to plaque on these cells in the presence of 5-bromo-deoxyuridine as well as by DNA-DNA hybridization to an appropriate radiolabeled probe comprising either LAV/HTLV III envelope or LAV/HTLV III gag sequences. Hybridization-positive plaques were purified, expanded and the resulting recombinant viruses were tested

for their ability to produce LAV/HTLV III envelope glycoproteins or core structural proteins. The LAV/HTLV III envelope related proteins expressed by the recombinant vaccinia viruses proved to be antigenic and immunogenic, and capable of eliciting both humoral and cell mediated immunity in sub-human primates. The LAV/HTLV III gag related proteins expressed by the recombinant vaccinia viruses were immunoreactive proteins that contained the major epitopes of authentic core proteins.

In other specific embodiments of the present invention, env or gag sequences of LAV/HTLV III were inserted into a plasmid so that a baculovirus polyhedrin promoter was positioned 5′ to the initiation methionine sequence (ATG) of the LAV/HTLV III gene followed by polyhedrin DNA sequences on the 3′ end. These plasmids were then co-transfected into cells together with wild-type baculovirus DNA, thus allowing the chimeric LAV/HTLV III gene flanked by additional AcNPV sequences to be recombined into the polyhedrin gene of the baculovirus. The cells were allowed to lyse and the resulting viruses plaqued. Recombinant virus was selected by visual inspection for lack of occlusion bodies or by DNA-DNA hybridization to radiolabeled LAV/HTLV III env or LAV/HTLV III gag probes. Recombinant plaques were purified and expanded, and the resulting recombinant viruses were screened for their ability to produce LAV/HTLV III related proteins by immunoprecipitation followed by analysis on SDS polyacrylamide gels. Infected cell lysates were tested for their ability to detect LAV/HTLV III antibodies in serum by ELISA.

4. DESCRIPTION OF THE FIGURES

FIG. 1 represents the integrated proviral genome structure of LAV/HTLV III. Hatched areas indicate regions of open reading frames. Such regions encoding the group-specific antigen, the reverse transcriptase and envelope

proteins are designated as gag, pol and env respectively. Overlapping open reading frames are shown by cross-hatched areas. Numbers refer to numbers of base pairs downstream from the capsite, where the viral transcript starts. Restriction sites are marked as follows: Bg, BglII; Ec, EcoRI; Hn, HindIII; Kp, KpnI; Ss, SstI.

FIG. 2 represents the nucleotide sequence of the LAV/HTLV III-specific region (EcoRI to SstI) present in plasmid pRS-3 DNA; the entire LAV/HTLV III envelope gene (nucleotide 5766 to 8349) is contained within the LAV/HTLV III insert. Restriction sites used in the construction of pv-env1, pv-env2 and pv-env5 are indicated. The entire amino acid sequence of the envelope gene, as deduced from the nucleotide sequence data, is also indicated.

FIG. 3 is a schematic representation of the construction of plasmids containing a portion of the LAV/HTLV III envelope protein coding sequence inserted downstream from a vaccinia virus promoter. The LAV/HTLV III envelope coding sequence is represented by the open bar and the vaccinia promoter by the shaded bar. The nucleotide sequence at the junction of the vaccinia promoter region and the LAV/HTLV III envelope coding sequence is indicated at the lower portion of the figure. The underlined sequences indicate the presumed initiating codons and the reading frame for the chimeric gene. Note that the third and fourth amino acids in the translated sequence (Pro-Val) of the recombinant pv-env2 correspond to amino acids number 43 and 44 of the LAV/HTLV III envelope coding sequence.

FIG. 4 is a schematic representation of the construction of plasmids containing the entire LAV/HTLV III envelope protein coding sequence inserted downstream from a vaccinia virus promoter. The vaccinia virus promoter is represented by the shaded bar. Plasmid pv-env5 containing the entire LAV/HTLV III envelope coding region was constructed in two stages. The 5′ and 3′ portions of the coding region were

first cloned into pGS20 separately to form pv-env1 which contains the amino coding terminus of the LAV/HTLV III envelope gene and pv-env2 which contains the carboxyl coding terminus of the LAV/HTLV III envelope gene. These two portions were rejoined at the StuI site as shown.

FIG. 5 is a schematic representation of the construction of plasmids containing the LAV/HTLV III envelope protein coding sequence, lacking the transmembrane (anchor) sequence, inserted downstream from a vaccinia virus promoter. Plasmid pv-env7 was constructed in two stages. The 5′ portion of pv-env5 was inserted into p26 to form an intermediate plasmid, pv-env5/26, which provided an in-frame translation termination sequence (TAA) resulting in a truncated env gene. The truncated env sequence, terminating with TAA, was then inserted into pG20 in order to construct pv-env7 in which the truncated env gene is flanked by vaccinia TK gene sequences.

FIG. 6 represents the construction and selection for recombinant vaccinia virus. Solid bars represent the thymidine kinase (TK) gene of vaccinia virus. This TK gene is also present in plasmid pv-env5 DNA, but in the plasmid the TK gene is interrupted by the chimeric gene consisting of the vaccinia 7.5K promoter (shaded bar) and the LAV/HTLV III envelope coding region (open bar). After cells are infected with vaccinia, the recombinant plasmid containing the TK gene interrupted by the LAV/HTLV III envelope gene is introduced into the infected cells. Recombinations which occur in TK sequences flanking the chimeric gene introduce the LAV/HTLV III envelope gene sequence into the vaccinia viral genome. The resultant recombinant virus containing the LAV/HTLV III envelope gene is TK^-.

FIG. 7 represents a characterization of the genome structures of recombinant vaccinia viruses containing the LAV/HTLV III env gene. FIG. 7A represents a restriction enzyme analysis of vaccinia recombinants v-env5 and v-env5NY as well as their respective parental vaccinia strains (WR and

v-NY, respectively). DNA fragments generated by restriction enzyme HindIII digests were resolved by electrophoresis on an agarose gel and stained with ethidium bromide in order to visualize the bands. FIG. 7B represents the Southern blot obtained after transfer of the resolved restriction fragments to nitrocellulose and hybridization to a nick-translated probe specific for LAV/HTLV III envelope sequences.

FIG. 8A represents a Western immunoblot analysis of the proteins expressed by vaccinia-LAV/HTLV III env recombinants. Proteins from the following sources were resolved on a 7-15% gradient SDS-polyacrylamide gel and electrotransferred to nitrocellulose paper: LAV/HTLV III virion proteins (LAV/HTLV III); wild-type vaccinia virus infected cells (WTvv); uninfected cells (mock); cells infected by recombinant viruses v-env2 (v-env2), or v-env5 (v-env5). Proteins immunoreactive to AIDS patient serum were detected through the action of peroxidase conjugated to anti-human IgG antibodies. LAV/HTLV III env gene products are indicated as gp150, gp110 and gp41. Molecular weight standards are expressed in kilodaltons.

FIG. 8B represents a Western immunoblot analysis of the proteins exprssed by vaccinia-LAV/HTLV III env recombinants in two cell types. Proteins derived from either BSC-40 or HeLa cells were resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and electrotransferred to nitrocellulose paper and reacted with pooled serum from LAV/HTLV III seropositive individuals; lanes 1 and 5 represent mock-infected cells; lanes 2 and 6 represent cells infected with wild type vaccinia virus; lanes 3 and 7 represent cells infected with v-env5; and lanes 4 and 8 represent cells infected with v-env2. Immunoreactive proteins were detected using protein A labeled with ^{125}I. LAV/HTLV III-env gene products are indicated as gp150, gp110 and gp41.

FIG. 9A represents the results of a radio-immunoprecipitation analysis of the proteins expressed in cells infected with either wild-type vaccinia virus (WTvv) or recombinant vaccinia virus (v-env2 and v-env5). Proteins were labeled with ^{35}S-methionine from 10-12 hours after infection. Labeled proteins in cell lysates were reacted with either control human serum (N) or AIDS patient serum (I) and the immune complexes were precipitated by <u>Staphylococcus aureus</u> protein A. Immunoprecipitated proteins were resolved by electrophoresis on a 15% SDS-polyacrylamide gel and detected by fluorography. Molecular weights are indicated in kilodaltons.

FIG. 9B represents the results of radioimmuno-precipitation analysis of ^{3}H-glucosamine labeled proteins expressed by the vaccinia-LAV/HTLV III recombinants of the invention. Hela cells were either mock infected (lanes 1 and 5), or infected with wild-type vaccinia virus (lanes 2 and 6), v-env5 (lanes 3 and 7) or v-env2 (lanes 4 and 8), and labeled with ^{3}H-glucosamine. Cell lysates were immunoprecipitated with either normal human serum (lanes 1-4) or pooled serum from LAV/HTLV III seropositive individuals (lanes 5-8) and protein A. The immunoprecipitated proteins were resolved by SDS-PAGE.

FIG. 9C represents the results of a "pulse-chase" radioimmunoprecipitation analysis of LAV/HTLV III envelope proteins expressed by vaccinia-LAV/HTLV III recombinants. Hela cells were infected with wild-type vaccinia virus, v-env5 or v-env2 as indicated, labeled with ^{35}S-methionine, and washed with chase-medium. At the following time intervals, the cells were washed, lysed and immunoprecipitated with pooled serum from LAV/HTLV III seropositive individuals and resolved by SDS-PAGE: 0 hour (lanes 1, 7, 13); 0.5 hour (lanes 2, 8, 14); 1 hour (lanes 3, 9, 15); 2 hours (lanes 4, 10, 16); 6 hours (lanes 5, 11, 17) and 12 hours (lanes 6, 12,

18). Lane M represents markers comprising ^{14}C labeled molecular weight standards.

FIG. 9D represents the results of a radioimmunoprecipitation analysis of the LAV/HTLV III envelope related proteins found in cells and in media from cells infected with the recombinant vaccinia-LAV/HTLV III viruses of the invention. Hela cells were either mock infected (lane 1), or infected with either wild-type vaccinia virus (lane 2) v-env5 (lane 3) or v-env2 (lane 4), and labeled with ^{35}S-methionine. The cells were separated from medium and lysed. The cell lysates (pellet) and the media (supe) were each immunoprecipitated using pooled serum from LAV/HTLV III seropositive individuals. The immunoprecipitated proteins were resolved by SDS-PAGE.

FIG. 9E represents the results of radioimmunoprecipitation analysis of LAV/HTLV III envelope related proteins in cells and in media from cells infected with recombinant vaccinia-LAV/HTLV III viruses of the invention. Recombinant v-env5 contains the entire LAV/HTLV III envelope coding sequence, whereas v-env7 contains the LAV/HTLV III envelope coding sequence lacking the transmembrane (anchor) region. Hela cells were either mock infected (lane 1) or infected with wild-type vaccinia virus (lane 2), v-env5 (lane 3) or v-env7 (land 4), and labeled with ^{35}S-methionine. The cells were separated from medium and lysed. The cell lysates (pellet) and the media (supernatant) were each immunoprecipitated using pooled serum from LAV/HTLV III seropositive individuals. The immunoprecipitated proteins were resolved by SDS-PAGE.

FIG. 10 represents a Western immunoblot analysis of serum samples from mice immunized with vaccinia-LAV/HTLV III recombinant viruses. Mice were immunized with v-env5 or v-env2 recombinant vaccinia virus. After 8 weeks, serum samples were reacted with LAV/HTLV III virion proteins which had been resolved by SDS-PAGE and electrotransferred to

nitrocellulose paper. A goat anti-mouse immunoglobulin conjugated to alkaline phosphatase was used to detect those LAV/HTLV III proteins which were recognized by the mouse sera. Lanes a to e represent serum samples from 5 individual mice inoculated with v-env5, and lanes f to k represent serum samples from 5 individual mice inoculated with v-env2. Pooled sera from LAV/HTLV III seropositive individuals (AIDS) and unimmunized C57Bl6J mice (NMS) were used as positive and negative controls, respectively. Positions of LAV/HTLV III envelope glycoproteins gp150, gp110, and gp41 are indicated.

FIG. 11 is a histogram representing the seroconversion of macaque monkeys vaccinated with recombinant vaccinia virus, v-env5. Four monkeys were inoculated by skin scarification with 2 x 10^8 pfu and four with 2 x 10^7 pfu of v-env5. One animal was inoculated with 2 x 10^7 pfu of a control vaccinia-herpes simplex gD recombinant (v-HSVgD1). Ten weeks after the primary inoculation all animals with the exception of No. 81 were given a second inoculation of 2 x 10^8 pfu of the same virus. Serum samples were collected prior to inoculation (open bars); 4 weeks after the primary inoculation (hatched bars); and 4 weeks after the second inoculation (solid bars). Seroconversion was assayed by ELISA using purified LAV/HTLV III virions as the target antigen.

FIG. 12 represents a Western immunoblot analysis of serum samples from the macaque monkeys immunized with vaccinia-LAV/HTLV III recombinant virus, v-env5, as described for FIG. 11. Serum samples were collected prior to the first inoculation (lane pre) and at 4 weeks after the second inoculation. Aliquots of serum were diluted 50-fold and reacted with LAV/HTLV III virion protein which had been resolved by SDS-PAGE and immobilized on nitrocellulose filters by electrotransfer using a protocol which provides for the optimal detection of two envelope glycoproteins, gp110 (FIG. 12A) and gp41 (FIG. 12B). LAV/HTLV III proteins

recognized by the macaque sera were detected by goat anti-human immunoglobulin conjugated with alkaline phosphatase. Pooled sera from LAV/HTLV III seropositive individuals (AIDS) were used as positive control. Authentic LAV/HTLV III virions (AIDS) were run as a control.

FIG. 13 represents a Western immunoblot analysis of serum samples from chimpanzees immunized with vacciniaNY-LAV/HTLV III recombinant virus, v-env5NY. Two chimpanzees were inoculated intradermally with 5 x 10^8 pfu of v-env5NY, while one animal was inoculated with the same dosage v-HSVgD1NY, a vaccinia-herpes simplex virus gD recombinant constructed from the same parental vaccinia-NY strain. All animals received a second inoculation 8 weeks after the primary inoculation. Serum samples were collected prior to immunization (lane 1), 8 weeks after the primary immunization (lane 2) and 2 weeks after the second immunization (lane 3). Aliquots of serum were diluted 50-fold and reacted with LAV/HTLV III virion proteins which had been resolved by SDS-PAGE and immobilized on nitrocellulose filters by electrotransfer, using a protocol which allowed optimal detection of gp41. LAV/HTLV III proteins recognized by the chimpanzee sera were detected by goat anti-human immunoglobulin conjugated with alkaline phosphatase. Pooled sera from LAV/HTLV III seropositive individuals (AIDS) was used as a positive control.

FIG. 14 represents the nucleotide sequence of the LAV/HTLV III- specific region (<u>SstI</u> to <u>KpnI</u>) present in plasmid pKS-5 DNA; the entire LAV/HTLV III gag gene (nucleotide 340 to 1835) is contained within the LAV/HTLV III insert. Restriction sites used in the construction of pAc-gag1 are indicated. The entire amino acid sequence of the gag gene, as deduced from the nucleotide sequence data, is also indicated.

FIG. 15 is a schematic representation of the construction of plasmid pAc-gag1 containing the entire

LAV/HTLV III gag protein coding sequence inserted downstream from an AcNPV promoter. The pAc610 cloning vector contains nucleotide sequences corresponding to the polyhedrin gene promoter, and includes 5′ leader sequences and 3′ polyhedrin sequences interrupted by cloning sites located downstream from the transcriptional start site. Cloning sites at position -8 are: EcoRI, SstI, SmaI (XmaI), BamHI, XbaI and PstI. SalI, AccI, and HincII are not unique. No sites for KpnI or BglII are present.

FIG. 16 schematically represents the construction and selection for recombinant AcNPV (Ac-gag1). The shaded bar indicates polyhedrin promoter and 5′ leader sequences. Solid bars represent the polyhedrin gene of baculovirus. Parts of this gene are present in plasmid pAc-gag1 DNA, interrupted by the LAV/HTLV III gag coding region (open bar). Cells are co-transfected with recombinant plasmid DNA containing portions of the polyhedrin gene interrupted by the LAV/HTLV III gag gene (pAc-gag1) together with wild-type AcNPV DNA. Recombinations which occur in the polyhedrin sequences flanking the chimeric gene introduce the LAV/HTLV III gag gene sequence into the AcNPV genome.

FIG. 17 represents a Northern blot analysis of RNA extracted from Spodoptera frugiperda cells infected with wild-type AcPNV and Ac-gag1 using LAV/HTLV III gag specific probes.

FIG. 18 represents the results of a radioimmuno-precipitation analysis of the proteins expressed in cells infected with recombinant baculovirus Ac-gag1. Proteins were labeled with [^{35}S] methionine for 2 hours at 24 hours (lane 1,2), 48 hours (lane 3,4) and 72 hours (lane 5,6) post-infection. Labeled proteins in cell lysates were reacted with either control human serum (lane 1,3,5) or AIDS patient serum (lane 2,4,6), and the immune complexes were precipitated by Staphylococcus aureus protein A. Immunoprecipitated proteins were resolved by electrophoresis

on a 10% SDS polyacrylamide gel and detected by fluorography. Molecular weights are indicated in kilodaltons.

FIG. 19 represents the results of a "pulse-chase" radioimmunoprecipitation analysis of the LAV/HTLV III gag related proteins found in cells infected with the recombinant AcNPV of the invention. Spodoptera frugiperda (Sf9) cells were infected with Ac-gag1 and pulse labeled for 5 minutes at 24 hours after infection. The cells were then washed with complete medium and incubated with complete medium for 0 hours (lane 1,2), 2 hours (lane 3,4), 4 hours (lane 5,6), and 8 hours (lane 7,8). At those times, cells were washed, lysed, and immunoprecipitated with pooled serum from LAV/HTLV III seropositive individuals (lane 2,4,6,8) or normal human serum (lane 1,3,5,7). Proteins were resolved by SDS polyacrylamide gel electrophoresis.

FIG. 20 schematically represents the construction of five plasmids, pv-gag1, pv-gag2, pv-gag3, pv-gag4 and pv-gag5 containing the vaccinia virus 7.5K promoter ligated to various lengths of the LAV/HTLV III genome containing gag-encoding sequences. The pGS62 cloning vector used in these constructions is identical to pGS20 (see FIG. 3) with the exception of having a unique EcoRI site located downstream from the unique SmaI site of pGS20.

FIG. 21 represents the results of a Western immunoblot analysis of the proteins expressed in cells infected with either recombinant vaccinia LAV/HTLV III viruses (v-gag1NY, v-gag2NY, v-gag3NY, v-gag4NY and v-gag5NY), the parental vaccinia virus (v-NY), and mock-infected cells (MOCK). Samples of purified LAV/HTLV III virions (LAV/HTLV III) were included as positive controls. Confluent BSC-40 cells were infected at a moi of 10. Infection was allowed to proceed for 12 hours at which time cells were harvested and lysed. Total cellular proteins were resolved by SDS-PAGE and immobilized on nitrocellulose by electrotransfer. The filters were reacted with either (1) AIDS patient serum (FIG.

21B) which was then detected using goat anti-human immunoglobulin conjugated to alkaline phosphatase; (2) mouse monoclonal antibodies that define gag proteins p25 and p18 (FIGS. 21A and 21C) which were then detected using goat anti-mouse immunoglobulin conjugated to alkaline phosphatase.

FIG. 22 schematically represents the construction of plasmid pAc-env5 containing the entire LAV/HTLV III envelope coding sequence inserted downstream from an AcNPV polyhedrin promoter. The pAc610 cloning vector is described in FIG. 15 above.

FIG. 23 represents the results of a radioimmunoprecipitation analysis of the proteins expressed in cells infected with recombinant baculovirus Ac-env5. Mock infected cells (MOCK) and the parental baculovirus strain (AcNPV) are included as controls. Proteins expressed by infected Sf9 cells were labeled with ^{35}S-methionine for 2 hours at 28 hours post infection. Labeled proteins in cell lysates were reacted with either AIDS patient serum or with mouse monoclonal antibodies that define gp110 or gp41, and the immune complexes were precipitated using Staphylococcus aureus protein A. Immunoprecipitated proteins were resolved by SDS-PAGE and detected by fluorography.

5. DETAILED DESCRIPTION OF THE INVENTION

The present invention is directed to viruses that produce peptides and proteins related to epitopes of LAV/HTLV III. The invention is also directed to peptides and proteins related to epitopes of LAV/HTLV III which can be produced using recombinant DNA methods or by chemical synthesis. The viruses or peptides and proteins of the present invention which are capable of eliciting a protective immune response may be used as immunogens in various vaccine formulations, including multivalent vaccine formulations, to protect against infection with LAV/HTLV III, the etiological agent of LAS and AIDS. Alternatively, the peptides or proteins of the

invention may be used as antigens in diagnostic immunoassays for the detection of patient antibodies specific for LAV/HTLV III. The peptides or proteins used in the immunoassays of the invention should be antigenic (i.e., reactive with patient antibodies for LAV/HTLV III) but need not be immunogenic (i.e., capable of eliciting an immune response). Moreover, the antigens used in the diagnostic immunoassay need not elicit a protective immune response in vivo.

According to one embodiment of the present invention, recombinant DNA techniques are used to insert nucleotide sequences encoding LAV/HTLV III epitopes into expression vectors that will direct the expression of the LAV/HTLV III sequences in appropriate host cells. These expression vector-host cell systems can be used to produce LAV/HTLV III related peptides and proteins in vitro in which case, the gene products can be purified from the cells in culture. The peptides or proteins which can elicit a protective immune response may be used as immunogens in subunit vaccine formulations. Alternatively, immunogenic or merely antigenic peptides and proteins of the invention can be used as antigens in immunoassays designed to detect patient antibodies specific for LAV/HTLV III. In an alternate approach for the production of the peptides and proteins of the invention, the amino acid sequence of these peptides and proteins may be deduced from the LAV/HTLV III nucleotide sequences contained in recombinants that express antigenic and/or immunogenic LAV/HTLV III related peptides and proteins. These peptides and proteins may then be chemically synthesized and used in synthetic subunit vaccine formulations (if immunogenic) or as antigens in diagnostic immunoassays (if antigenic and/or immunogenic).

Where the expression vector is a virus that directs the expression of an immunogen related to an epitope of LAV/HTLV III that is capable of eliciting a protective immune response, the virus itself can be formulated as a vaccine.

Infectious recombinant viruses that do not cause disease in the host can be used in live virus vaccine preparations which provide for substantial immunity. Alternatively, inactivated virus vaccines can be prepared using "killed" viruses. In addition, multivalent vaccines containing two or more epitopes of LAV/HTLV III or an epitope of LAV/HTLV III as well as those of other disease causing agents may be prepared.

The method of the invention may be divided into the following stages solely for the purpose of description: (a) isolation of a gene, or gene fragment, encoding LAV/HTLV III viral proteins, (b) insertion of the gene or gene fragment into an expression vector, (c) identification and growth of the recombinant expression vector in a host system which is capable of replicating and expressing the gene, (d) identification and purification of the gene product, (e) determination of the immunopotency of the product and (f) formulation of a vaccine.

In specific embodiments of the present invention, we describe the construction of recombinant vaccinia viruses and baculoviruses containing the envelope gene of LAV/HTLV III which direct the expression of proteins immunologically related to the envelope proteins of LAV/HTLV III in tissue culture cells infected by the recombinant viruses. In other embodiments of the present invention, we describe the construction of recombinant vaccinia viruses and baculoviruses containing the gag gene of LAV/HTLV III which direct the expression of proteins immunologically related to the core structural proteins of LAV/HTLV III in tissue culture cells infected by the recombinant viruses. However, the compositions and methods described herein are not limited to the construction of recombinant viruses expressing LAV/HTLV III envelope or gag related proteins and may be used to construct recombinants in any expression vector system for

the production of polypeptides related to antigens of any etiological agent of AIDS.

For clarity of discussion, the entire method will be discussed in terms of the LAV/HTLV III envelope and gag genes. The same technique, however, may be applied in an analogous fashion to construct recombinant expression vectors and to produce polypeptides related to any of the proteins of LAV/HTLV III as well as those of related viruses. Such proteins include but are not limited to the gene products of LAV/HTLV III such as gag, pol and env and at least four additional genes named to date: sor, tat, 3'-orf and a gene variously named art or trs (see, Fisher et al., 1986, Science 233:655-659).

5.1. ISOLATION OF GENES OR GENE FRAGMENTS ENCODING LAV/HTLV III VIRAL PROTEINS

Isolation of the LAV/HTLV III genes involves first isolating DNA fragments which contain the desired gene sequences such as envelope or gag. As previously explained, LAV/HTLV III has an RNA genome, therefore, the corresponding DNA which encodes the LAV/HTLV III gene can be obtained either (a) by cDNA cloning of RNA isolated from purified LAV/HTLV III virions (b) by cDNA cloning of poly[A]-containing RNA obtained from LAV/HTLV III-infected cells or (c) by cloning genomic DNA purified from LAV/HTLV III infected cells. Hereinafter, DNA encoding LAV/HTLV III genes will be referred to as LAV/HTLV III DNA.

In order to generate LAV/HTLV III DNA fragments, the LAV/HTLV III DNA may be cleaved at specific sites using various restriction enzymes. Alternatively one may use DNase in the presence of manganese to fragment the DNA, or the DNA can be physically sheared, as for example, by sonication. The linear DNA fragments can then be separated according to size by standard techniques, including, but not limited to,

agarose and polyacrylamide gel electrophoresis and column chromatography.

Any restriction enzyme or combination of restriction enzymes may be used to generate LAV/HTLV III DNA fragment(s) containing the envelope or gag sequences provided the enzymes do not destroy the antigenicity of the protein gene product. For example, the antigenic site of a protein can consist of from about 7 to about 14 amino acids. Thus, a protein of the size of the envelope peptide precursor (approximately 97,000 daltons) may have many discrete antigenic sites, possibly thousands considering overlapping sequences, secondary and tertiary structure considerations, and processing events such as acetylation, glycosylation or phosphorylation. Therefore, many partial envelope polypeptide gene sequences could code for an antigenic site. Consequently, many restriction enzyme combinations may be used to generate DNA fragments which, when inserted into an appropriate vector, are capable of directing the production of envelope specific amino acid sequences comprising different antigenic determinants.

Once the DNA fragments are generated, identification of the specific DNA fragment containing the desired LAV/HTLV III gene may be accomplished in a number of ways. Firstly, it is possible to sequence the DNA fragments corresponding to the entire LAV/HTLV III genome and then identify the fragment containing the envelope protein gene or the gag gene sequence based upon a comparison of the predicted amino acid sequence to the amino acid sequence of the envelope protein or gag core proteins, respectively. Secondly, once the entire genomic sequence has been determined, the large open reading frames can be ordered from 5′ to 3′. As the genomic organization of all retroviruses examined to date is 5′-gag-pol-env-3′, the large open reading frame closest to the 3′ end will most likely code for the envelope gene whereas the large open reading frame closest to the 5′-end will most likely code for the gag gene. Thirdly, probable

identification of a specific gene can be accomplished by recognition of homology to other known retroviral genes, either by nucleic acid hybridization analyses or sequence comparisons if the sequences are known.

Alternatively, the fragment containing the envelope or gag gene may be identified by mRNA selection. In this procedure the LAV/HTLV III DNA fragments are used to isolate complementary mRNAs by hybridization. Immunoprecipitation analysis of the in vitro translation products of the isolated mRNAs identifies the mRNA and, therefore, the complementary LAV/HTLV III DNA fragments that contain the envelope or gag protein sequences. Finally, specific mRNAs may be selected by adsorption of polysomes isolated from LAV/HTLV III-infected cells to immobilized antibodies directed against envelope or gag proteins. A radiolabelled envelope protein cDNA (complementary DNA) can be synthesized using the selected mRNA (from the adsorbed polysomes) as a template. The radiolabeled mRNA or cDNA may then be used as a probe to identify the LAV/HTLV III DNA fragments containing envelope or gag gene seqences. Alternatives to isolating the envelope or gag gene include but are not limited to, chemically synthesizing the gene sequence itself (provided the sequence is known) or making cDNA to the mRNA which encodes the envelope or gag gene.

Once identified and isolated, the LAV/HTLV III DNA fragment containing the sequences of interest may be first inserted into a cloning vector such as a plasmid cloning vector which is used to transform appropriate host cells in order to replicate the DNA so that many copies of the LAV/HTLV III sequences of interest are generated. This can be accomplished by ligating the LAV/HTLV III DNA fragment into a cloning vector which has complementary cohesive termini. However, if the complementary restriction sites used to fragment the LAV/HTLV III DNA are not present in the cloning vector, the ends of the DNA molecules may be

modified. Such modifications include producing blunt ends by digesting back single-stranded DNA termini or by filling the single-stranded termini so that the ends can be blunt-end ligated. Alternatively, any site desired may be produced by ligating nucleotide sequences (linkers) onto the DNA termini; these ligated linkers may comprise specific chemically synthesized oligonucleotides encoding restriction site recognition sequences. According to other methods, the cleaved vector and the LAV/HTLV III DNA fragment may be modified by homopolymeric tailing.

Transformation of host cells with recombinant DNA molecules that incorporate the isolated gene, cDNA or synthesized DNA sequence enables generation of multiple copies of the gene. Thus, the gene may be obtained in large quantities by growing transformants, isolating the recombinant DNA molecules from the transformants and, when necessary, retrieving the inserted gene from the isolated recombinant DNA.

If the ultimate goal is to insert the gene into virus expression vectors such as vaccinia virus or adenovirus, the recombinant DNA molecule that incorporates the LAV/HTLV III gene can be modified so that the gene is flanked by virus sequences that allow for genetic recombination in cells infected with the virus so that the gene can be inserted into the viral genome.

The entire LAV/HTLV III genome has been cloned and sequenced by Wain-Hobson et al. (Wain-Hobson, S., et al., 1985, Cell 40:9). One clone, referred to as lambda J19 contained a 9.2 kilobase pair DNA fragment of an LAV/HTLV III genomic sequence inserted into the HindIII site of lambda L 47.1.

A particularly useful subclone of lambda J19 containing the LAV/HTLV III envelope gene is pRS-3 which consists of a 3,840 base pair EcoRI to SstI fragment of the LAV/HTLV III nucleotide sequence inserted into the EcoRI and SstI site of

pUC18. The LAV/HTLV III specific DNA contained in pRS-3 is from the EcoRI site located at nucleotide 5289 to the SstI site located at nucleotide 9129 on the LAV/HTLV III genome (Wain-Hobson, S., et al., 1985, Cell 40:9); see FIG. 2 which depicts the LAV/HTLV III nucleotide sequence contained in pRS-3. However, due to the degeneracy of nucleotide coding sequences, other DNA sequences which encode substantially the same amino acid sequence as depicted in FIG. 2 may be used in the practice of the present invention for the cloning of the envelope gene of LAV/HTLV III. These include but are not limited to nucleotide sequences comprising all or portions of the envelope nucleotide sequence depicted in FIG. 2 which are altered by the substitution of different codons that encode the same or a functionally equivalent amino acid residue within the sequence (for example, an amino acid of the same polarity) thus producing a silent change.

Particularly useful subclones of lambda J19 containing the LAV/HTLV III gag gene are pKS-5 and pSS5. The pKS5 plasmid consists of a 3,148 base pair SstI to KpnI LAV/HTLV III fragment in pUC18. The LAV/HTLV III specific DNA contained in pKS-5 is from the SstI site located at nucleotide 224 to the KpnI site located at nucleotide 3,372 on the LAV/HTLV III genome. The pSS-5 plasmid consists of a 5.1 kbp SstI to SalI LAV/HTLV III fragment in pUC 18. the LAV/HTLV III specific DNA contained in pSS-5 is from the SstI site located at nucleotide 224 to the SalI site located at nucleotide 5331 on the LAV/HTLV III genome. (Wain-Hobson, S., et al., 1985, Cell 40:9) see FIG. 14 which depicts the LAV/HTLV III nucleotide sequence contained in pKS-5 and PSS-5. However, due to the degeneracy of nucleotide coding sequences, other DNA sequences which encode substantially the same amino acid sequence as depicted in FIG. 14 may be used in the practice of the present invention for the cloning of the gag gene of LAV/HTLV III. These include but are not limited to nucleotide sequences comprising all or portions of

the gag nucleotide sequence depicted in FIG. 14 which are altered by the substitution of different codons that encode the same or a functionally equivalent amino acid residue within the sequence (for example, an amino acid of the same polarity) thus producing a silent change.

5.2. INSERTION OF THE LAV/HTLV III PROTEIN CODING SEQUENCES INTO EXPRESSION VECTORS

The nucleotide sequence coding for a LAV/HTLV III protein such as envelope, gag, or a portion thereof, is inserted into an appropriate expression vector, i.e. a vector which contains the necessary elements for the transcription and translation of the inserted protein-coding sequence. A variety of host-vector systems may be utilized to express the protein-coding sequence. These include but are not limited to mammalian cell systems infected with virus (e.g. vaccinia virus, adenovirus, etc.); insect cell systems infected with virus (e.g. baculovirus); microorganisms such as yeast containing yeast vectors or bacteria transformed with bacteriophage DNA, plasmid DNA or cosmid DNA. The expression elements of these vectors vary in their strength and specificities. Depending on the host-vector system utilized, any one of a number of suitable transcription and translation elements may be used. For instance, when cloning in mammalian cell systems, promoters isolated from the genome of mammalian cells, (e.g. mouse metallothionien promoter) or from viruses that grow in these cells, (e.g. vaccinia virus 7.5K promoter) may be used. Promoters produced by recombinant DNA or synthetic techniques may also be used to provide for transcription of the inserted sequences.

Specific initiation signals are also required for efficient translation of inserted protein coding sequences. These signals include the ATG initiation codon and adjacent sequences. In cases where the entire LAV/HTLV III envelope or gag gene including its own initiation codon and adjacent

sequences are inserted into the appropriate expression vectors, no additional translational control signals may be needed. However, in cases where only a portion of the envelope or gag coding sequence is inserted, exogenous translational control signals, including the ATG initiation codon must be provided. The initation codon must furthermore be in phase with the reading frame of the protein coding sequences to ensure translation of the entire insert. These exogenous translational control signals and initiation codons can be of a variety of origins, both natural and synthetic.

Any of the methods previously described for the insertion of DNA fragments into a vector may be used to construct expression vectors containing a chimeric gene consisting of appropriate transcriptional/translational control signals and the protein coding sequences. These methods may include _in vitro_ recombinant DNA and synthetic techniques and _in vivo_ recombinations (genetic recombination).

In the particular embodiments detailed in the examples of the present invention, vaccinia virus and baculovirus were chosen as the expression vectors. However, the invention is not limited to the use of vaccinia virus or baculovirus. As previously explained, the expression vectors which can be used include, but are not limited to the following vectors or their derivatives: human or animal viruses such as vaccinia viruses or adenoviruses; insect viruses such as baculoviruses; yeast vectors; bacteriophage vectors, and plasmid and cosmid DNA vectors to name but a few.

In cases where an adenovirus is used as an expression vector, the LAV/HTLV III gene is ligated to an adenovirus transcriptional/translational control complex, _e.g._, the late promoter and tripartite leader sequences. This chimeric gene is then inserted in the adenovirus genome by _in vitro_ or _in vivo_ recombination. Insertion in a non-essential region of the viral genome (_e.g._, region E1 or E3) will result in a

recombinant virus that is viable and capable of expressing the LAV/HTLV III related protein in infected hosts. Presently, there are two strains of adenovirus (types 4 and 7) approved and used as vaccines for military personnel. They are prime candidates for use as vectors to express LAV/HTLV III genes.

In addition, a host cell strain may be chosen which modulates the expression of the inserted sequences, or modifies and processes the chimeric gene product in the specific fashion desired. Expression from certain promoters can be elevated in the presence of certain inducers, (e.g. zinc and cadmium ions for metallothionein promoters). Therefore, expression of the genetically engineered LAV/HTLV III protein may be controlled. This is important if the protein product of the cloned foreign gene is lethal to host cells. Furthermore, modifications (e.g. glycosylation) and processing (e.g. cleavage) of protein products are important for the function of the protein. Different host cells have characteristic and specific mechanisms for the post-translational processing and modification of proteins. Appropriate cell lines or host systems can be chosen to ensure the correct modification and processing of the foreign protein expressed.

In two particular embodiments detailed in the examples of the present invention, we have ligated either LAV/HTLV III envelope coding sequences, both in its complete form and portions thereof, or LAV/HTLV III gag coding sequences to the 7.5K promoter of vaccinia virus to form chimeric genes in various plasmids. The chimeric genes in these plasmids were flanked by additional vaccinia virus sequences homologous to the vaccinia virus TK gene. The construction of the chimeric genes involved the use of both natural and synthetic nucleotides encoding control signals for transcription and translation of the LAV/HTLV III env or the LAV/HTLV III gag sequences. These chimeric genes were then introduced into

vaccinia virus expression vectors through *in vivo* recombination between the homologous TK region present on both the plasmid vector and vaccinia viral genome. These recombinant viruses containing the chimeric gene were used as expression vectors to produce LAV/HTLV III envelope related proteins or LAV/HTLV III gag related proteins.

In other particular embodiments detailed in the examples of the present invention, we have ligated either LAV/HTLV III envelope coding sequences or LAV/HTLV III gag coding sequences to the polyhedrin promoter of *Autographa californica* nuclear polyhedrosis virus (AcNPV) to form chimeric genes in various plasmids. The chimeric genes in these plasmids were flanked by additional AcNPV sequences. The construction of the chimeric genes involved the use of natural nucleotides encoding control signals for transcription and translation of the LAV/HTLV III gag or env sequences. The chimeric genes were then introduced into AcNPV expression vectors through *in vivo* recombination between the homologous DNA present on both the plasmid vector and AcNPV genome. These recombinant viruses containing the chimeric genes were used as expression vectors to produce LAV/HTLV III envelope related proteins or LAV/HTLV III gag related proteins.

5.3. IDENTIFICATION OF RECOMBINANT EXPRESSION VECTORS CAPABLE OF REPLICATING AND EXPRESSING THE INSERTED GENE

Expression vectors containing foreign gene inserts can be identified by three general approaches: (a) DNA-DNA hybridization, (b) presence or absence of "marker" gene functions, and (c) expression of inserted sequences. In the first approach, the presence of a foreign gene inserted in an expression vector can be detected by DNA-DNA hybridization using probes comprising sequences that are homologous to the foreign inserted gene. In the second approach, the

recombinant vector/host system can be identified and selected based upon the presence or absence of certain "marker" gene functions (e.g. thymidine kinase activity, resistance to antibiotics, transformation phenotype, occlusion body formation in baculovirus etc.) caused by the insertion of foreign genes in the vector. For example, if the LAV/HTLV III gene is inserted within the marker gene sequence of the vector, recombinants containing the LAV/HTLV III insert can be identified by the absence of the marker gene function. In the third approach, recombinant expression vectors can be identified by assaying the foreign gene product expressed by the recombinant. Such assays can be based on the physical, immunological, or functional properties of the gene product.

Once a particular recombinant DNA molecule is identified and isolated, several methods may be used to propagate it, depending on whether such a recombinant constitutes a self-replicating unit (a replicon). A self replicating unit, e.g. plasmids, viruses, cells etc., can multiply itself in the appropriate cellular environment and growth conditions. Recombinants lacking a self-replicating unit will have to be integrated into a molecule having such a unit in order to be propagated. For example, certain plasmid expression vectors upon introduction into a host cell need to be integrated into the cellular chromosome to ensure propagation and stable expression of the recombinant gene. Once a suitable host system and growth conditions are established, recombinant expression vectors can be propagated and prepared in quantity.

In particular embodiments of the invention detailed in the examples, chimeric genes containing the env or gag coding sequence of LAV/HTLV III were inserted into the TK gene of the vaccinia virus genome, thereby converting the virus into TK^-, i.e. destroying the ability of the virus to make thymidine kinase. Such recombinants were selected by their ability to grow in media containing 5-bromo-deoxyuridine, a

nucleoside analog that is lethal to TK^+ cells but not to TK^- cells. Recombinants were further identified by DNA-DNA hybridization, using LAV/HTLV III env specific probes or LAV/HTLV III gag specific probes. TK^- recombinant virus was isolated by plaque-purification and stocks were prepared from infected tissue culture cells.

In other particular embodiments of the invention detailed in the examples, chimeric genes containing the env or gag coding sequence of LAV/HTLV III were inserted into the polyhedrin gene of the AcNPV genome, thereby converting the virus to a non-occlusion phenotype. Such recombinants were selected visually by their lack of occluded virus particles. Recombinants were further identified by Northern blot analysis using LAV/HTLV III env specific probes or LAV/HTLV III gag specific probes. Recombinant virus was isolated by plaque-purification and stocks were prepared from infected tissue culture cells.

5.4. IDENTIFICATION AND PURIFICATION OF THE EXPRESSED GENE PRODUCT

Once a recombinant which expresses the LAV/HTLV III gene is identified, the gene product should be analyzed. This can be achieved by assays based on the physical, immunological or functional properties of the product. Immunological analysis is especially important where the ultimate goal is to use the gene products or recombinant viruses that express such products in vaccine formulations and/or as antigens in diagnostic immunoassays.

A variety of antisera are available for analyzing immunoreactivity of the product, including but not limited to serum derived from LAS or AIDS patients and polyvalent antisera directed against the LAV/HTLV III virus, the viral envelope protein, or core proteins encoded by the gag gene. The immunogenic molecules include analogs produced in bacterial systems, or synthetic peptides containing antigenic

determinants of the LAV/HTLV III envelope or core antigens. Identification of the peptides and proteins described in this invention is based on two requirements. First, the LAV/HTLV III related protein should be produced only in recombinant virus infected cells. Second, the LAV/HTLV III related protein should be immunoreactive to the serum of AIDS patients or to a variety of antibodies directed against LAV/HTLV III envelope or core proteins or their analogs and derivatives.

The protein should be immunoreactive whether it results from the expression of the entire gene sequence, a portion of the gene sequence or from two or more gene sequences which are ligated to direct the production of fusion proteins. This reactivity may be demonstrated by standard immunological techniques, such as radioimmunoprecipitation, radioimmune competition, or immunoblots.

Once the LAV/HTLV III related protein is identified, it may be isolated and purified by standard methods including chromatography (e.g. ion exchange, affinity, and sizing column chromatography), centrifugation, differential solubility, or by any other standard technique for the purification of proteins.

Alternatively, once an immunoreactive LAV/HTLV III related protein produced by a recombinant is identified, the amino acid sequence of the immunoreactive protein can be deduced from the nucleotide sequence of the chimeric gene contained in the recombinant. As a result, the protein can be synthesized by standard chemical methods known in the art (e.g., see Hunkapiller, M. et al., 1984, Nature 310: 105-111).

In particular embodiments of the present invention such peptides, whether produced by recombinant DNA techniques or by chemical synthetic methods, include but are not limited to all or part of the amino acid sequences substantially as depicted in FIG. 2, or as depicted in FIG. 14, including

altered sequences in which functionally equivalent amino acid residues are substituted for residues within the sequence resulting in a silent change. For example, one or more amino acid residues within the sequence can be substituted by another amino acid of a similar polarity which acts as a functional equivalent, resulting in a silent alteration. Substitutes for an amino acid within the sequence may be selected from other members of the class to which the amino acid belongs. For example, the non polar (hydrophobic) amino acids include alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan and methionine. The polar neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine, and glutamine. The positively charged (basic) amino acids include arginine, lysine and histidine. The negatively charged (acidic) amino acids include aspartic and glutamic acid.

5.5. DETERMINATION OF THE IMMUNOPOTENCY OF THE RECOMBINANT PRODUCT

Immunopotency of the LAV/HTLV III related product can be determined by monitoring the immune response of test animals following immunization with the purified protein or synthetic peptide or protein. In cases where the LAV/HTLV III related protein is expressed by an infectious recombinant virus, the recombinant virus itself can be used to immunize test animals. Test animals may include mice, rabbits, chimpanzees and eventually human subjects. Methods of introduction of the immunogen may include oral, intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, intranasal or any other standard routes of immunizations. The immune response of the test subjects can be analysed by three approaches: (a) the reactivity of the resultant immune serum to authentic LAV/HTLV III viral antigens, as assayed by known techniques, e.g., enzyme linked immunosorbant assay (ELISA), immunoblots, radioimmunoprecipitations, etc., (b) the ability of the

immune serum to neutralize LAV/HTLV III infectivity <u>in vitro</u> (Robert-Guroff, M., 1985, Nature 316: 72-74) (for anti-envelope antibody detection), and (c) protection from LAV/HTLV III infection and/or attenuation of infectious symptoms in immunized animals (Francis, D.P., 1984, Lancet 2:1276-1277; Gujdusek, D.C., 1985, Lancet 1:55-56).

5.6. FORMULATION OF A VACCINE

The purpose of this embodiment of the invention is to formulate a vaccine in which the immunogen is related to an LAV/HTLV III epitope or which contains a recombinant virus which expresses an immunogen that elicits a protective response against LAV/HTLV III virus infections for the prevention of LAS or AIDS. Additionally, multivalent vaccine formulations can be prepared which contain more than one immunogenic LAV/HTLV III related determinant. Such vaccines include, but are not limited to those containing LAV/HTLV III envelope related epitopes formulated alone or in combination with other LAV/HTLV III epitopes such as the gag related epitopes. Such multivalent vaccines containing both env- and gag-encoded epitopes may be significant in preventing the development of AIDS. Individuals who are symptom-free appear to make anti-gag antibodies more often than do sick patients, while both groups of patients make antibodies directed against envelope determinants (Science, 1986, 233:419). Studies have also shown that AIDS patients in early symptom-free stages make antibodies against both envelope and core (gag) proteins. As the disease progresses and symptoms appear, the anti-gag antibodies are reduced, while anti-envelope antibodies remain (Science, 1986, 233:282). Thus inclusion of gag-related epitopes in vaccine formulations, the production of which is described as a specific embodiment of the present invention, may be significant in immunoprophylaxis or immunotherapy of LAV/HTLV III related disease.

Multivalent vaccines can be formulated to contain the LAV/HTLV III gene products and/or recombinant viruses expressing the chimeric gene products. These can also be used in combination with other immunogens for the prevention of LAS or AIDS and other diseases. Examples of various formulations are discussed below.

5.6.1. VIRAL VACCINE FORMULATIONS

Either a live recombinant viral vaccine or an inactivated recombinant viral vaccine can be formulated. The choice depends upon the nature of the recombinant virus used to express LAV/HTLV III related epitopes. Where the recombinant virus is infectious to the host to be immunized but does not cause disease, a live vaccine is preferable because multiplication in the host leads to a prolonged stimulus of similar kind and magnitude to that occurring in natural subclinical infections, and therefore, confers substantial long-lasting immunity. The infectious recombinant virus, upon introduction into a host animal, can express LAV/HTLV III related proteins from its chimeric gene and thereby stimulate an immune response against LAV/HTLV III antigens. In cases where such immune response is protective against subsequent LAV/HTLV III challenge, the live recombinant virus by itself may be used as a preventative vaccine against AIDS virus infection. Production of such recombinant virus to be used in these formulations may involve both in vitro (e.g. tissue culture cells) and in vivo (e.g. natural host) systems. Vaccinia virus recombinants are particularly useful in this regard. Conventional methods for the preparation and formulation of smallpox vaccine may be adapted for the formulation of live recombinant virus vaccine (for example, see Vaccinia Viruses and Factors for Vaccine Antigens, Ed. Quinnan, G.V., Elsevier, 1985, pp. 109-116).

Multivalent live virus vaccines can be prepared from a single or a few infectious recombinant viruses that express

several LAV/HTLV III/HTLV related epitopes. The vaccine may also include viruses that express epitopes of organisms that cause other diseases, in addition to the epitopes of LAV/HTLV III. For example, a vaccinia virus (which can accommodate approximately 35 kilobase pairs of foreign DNA) can be engineered to contain coding sequences for both envelope and gag related epitopes of LAV/HTLV III. The virus may also encode other epitopes in addition to those for LAV/HTLV III. Such a recombinant virus itself can be used as the immunogen in a multivalent vaccine. Alternatively, a mixture of vaccinia or other viruses, each capable of directing the expression of a different gene coding for different epitopes of LAV/HTLV III and/or other disease-causing organisms can be formulated in a multivalent vaccine.

Whether or not the recombinant virus is infectious to the host to be immunized, an inactivated vaccine formulation may be prepared. Inactivated vaccines are "dead" in the sense that their infectivity has been destroyed, usually by treatment with formaldehyde. Ideally, the infectivity of the virus is destroyed without affecting the capsid or envelope proteins which carry the immunogenicity of the virus. In order to prepare inactivated vaccines, large quantities of the recombinant virus must be grown in culture in order to provide the necessary quantity of relevant antigens. A mixture of inactivated viruses which express different epitopes may be used for the formulation of "multivalent" vaccines. In some instances this may be preferable to live vaccine formulations because of potential difficulties with mutual interference of live viruses administered together. In either case, the inactivated recombinant virus or mixture of viruses should be formulated with a suitable adjuvant in order to enhance the immunological response to their antigens. Suitable adjuvants include, but are not limited to, mineral gels, e.g. aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols,

polyanions, peptides, oil emulsions, and potentially useful human adjuvants such as BCG (bacille Calmette-Guerin) and corynebacterium parvum.

Many methods may be used to introduce the vaccine formulations described above; these include but are not limited to oral, intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous and intranasal routes. When a live recombinant virus vaccine formulation is used, it may be introduced via the natural route of infection of the parent wild-type virus which was used to make the recombinant virus in the vaccine formulation.

5.6.2. <u>SUBUNIT VACCINE FORMULATIONS</u>

In an alternative to viral vaccines, the LAV/HTLV III related protein itself may be used as an immunogen in subunit vaccine formulations, which may be multivalent. As previously explained, subunit vaccines comprise solely the relevant immunogenic material necessary to immunize a host. Accordingly, the LAV/HTLV III related protein(s) may be purified from recombinants that express the LAV/HTLV III epitopes. Such recombinants include any of the previously described virus-infected cultured cells, bacterial transformants, yeast transformants or virus-infected insects that express the LAV/HTLV III epitopes (see Sections 5.2, 5.3 and 5.4). In another embodiment of the present invention, the LAV/HTLV III related peptides or proteins may be chemically synthesized. To this end, the amino acid sequence of such a peptide or protein can be deduced from the nucleotide sequence of the chimeric gene which directs its expression (see Section 5.4).

Whether the immunogens are purified from recombinants or chemically synthesized, the final product may be adjusted to an appropriate concentration and formulated with any suitable vaccine adjuvant and packaged for use. Suitable adjuvants include, but are not limited to: mineral gels, e.g.,

aluminum hydroxide; surface active substances such as lysolecithin, pluronic polyols; polyanions; peptides; oil emulsions; and potentially useful human adjuvants such as BCG (bacille Calmette-Guerin) and corynebacterium parvum. The immunogen may also be incorporated into liposomes, or conjugated to polysaccharides and/or other polymers for use in a vaccine formulation.

In instances where the LAV/HTLV III related peptide or protein is a hapten, i.e., a molecule that is antigenic in that it can react selectively with cognate antibodies, but not immunogenic in that it cannot elicit an immune response, the hapten may be covalently bound to a carrier or immunogenic molecule; for instance, a large protein such as protein serum albumin will confer immunogenicity to the hapten coupled to it. The hapten-carrier may be formulated for use as a vaccine.

5.6.3 PASSIVE IMMUNITY AND ANTI-IDIOTYPIC ANTIBODIES

Instead of actively immunizing with viral or subunit vaccines, it may be possible to confer short-term protection to a host by the administration of pre-formed antibody directed against an epitope or epitopes of LAV/HTLV III. Accordingly, the vaccine formulations described above can be used to produce antibodies for use in passive immunotherapy. Human immunoglobulin is preferred in human medicine because a heterologous immunoglobulin will provoke an immune response to its foreign immunogenic components. Such passive immunization could be used on an emergency basis for immediate protection of unimmunized individuals exposed to special risks, e.g., those exposed to contact with AIDS patients, for instance, in hospitals and other health-care facilities. Alternatively, these antibodies can be used in the production of antiidiotypic antibody, which in turn can be used as an antigen to stimulate an immune response against LAV/HTLV III epitopes.

5.7. IMMUNOASSAYS

The LAV/HTLV III related peptides and proteins of the present invention may be used as antigens in immunoassays for the detection of antibodies to LAV/HTLV III in various patient tissues and body fluids as well as blood in blood banks and hospitals. To this end, the antigens of the present invention may be used in any immunoassay system known in the art including but not limited to competitive and non-competitive assay systems using techniques such as radioimmunoassays, ELISA (enzyme linked immunosorbent assay), "sandwich" immunoassays, precipitin reactions, gel diffusion precipitin reactions, immunodiffusion assays, agglutination assays, complement-fixation assays, immunoradiometric assays, fluorescent immunoassays, protein A immunoassays and immunoelectrophoresis assays, to name but a few.

The immunoassays of the present invention can be used to screen blood in blood banks and patients for exposure to LAV/HTLV III and to monitor patients who are being treated for LAS or AIDS. Any peptide or protein related to an antigen of LAV/HTLV III can be used in the immunoassay in accordance with the invention. Such antigens include but are not limited to peptides and proteins related to the gene products of env, gag, pol and the four additional genes named to date: sor, tat, 3'-orf and art (or trs). In a particular embodiment of the present invention, a panel of the antigens may be used in an immunoassay to screen patients for a profile of antibodies directed against different epitopes of LAV/HTLV III. In another embodiment of the invention, patients who have been vaccinated with a vaccine formulation of the present invention can be monitored to detect subsequent exposure to LAV/HTLV III. In such cases, the antigen used in the immunoassay is preferably a peptide or protein of the invention which was not an immunogen of the vaccine formulation that was used to vaccinate the patient, because such vaccinated individuals will be seropositive for

the particular epitope used in the vaccine formulation and, as a result, would test positive regardless of exposure to the virus. For example, if a patient was vaccinated with a LAV/HTLV III envelope related epitope of the present invention, that patient should be screened for LAV/HTLV III infection using any other non-envelope LAV/HTLV III related protein in order to determine the presence of patient antibodies specific for LAV/HTLV III. Thus, a patient vaccinated with an envelope-related epitope can be screened for antibodies specific for LAV/HTLV III core antigens, or other antigens of LAV/HTLV III including but not limited to the sor and 3'-orf gene products and the like.

In an alternative embodiment, the LAV/HTLV III-related peptides and proteins of the invention can be used in competitive immunoassays, to detect and quantitate the presence of LAV/HTLV III-HTLV III encoded proteins in patient blood, serum, etc.

6. EXAMPLE: VACCINIA ENVELOPE RECOMBINANTS

In the following examples, various plasmid vectors were constructed containing chimeric genes comprising LAV/HTLV III envelope coding sequences located downstream with respect to the transcriptional control sequences of vaccinia virus.

These chimeric genes containing the vaccinia promoter and the LAV/HTLV III envelope coding sequence were inserted into the genome of vaccinia virus through in vivo recombination. Such recombinant viruses were identified and purified, and viral stocks were prepared from infected tissue culture cells. Immunoreactive LAV/HTLV III envelope related proteins were shown to be produced by these recombinant vaccinia viruses in vitro. These recombinants were tested in experimental animals for their ability to elicit neutralizing or protective immune responses and for their use as a vaccine against AIDS. A detailed description of each step in this

embodiment of the invention is presented in the subsections below.

6.1. GENERAL PROCEDURES

6.1.1. CELLS AND VIRUSES

African green monkey kidney cells (strain BSC-40, a continuous line of African Green Monkey Cells derived from BSC-1 cells, ATCC No. CCL26) were obtained from R. Condit (Associate Professor, Department of Biochemistry, State University of New York, Buffalo, N.Y.) and were propagated in Dulbecco modified Eagle's medium (DMEM, Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum and 100 units per ml each of penicillin and streptomycin. Human 143 TK^- cells (Rhim, J.S. et al., 1975, Intl. J. Cancer 15: 23-29) were obtained from M. Botchan (Professor, Department of Molecular Biology, University of California, Berkley, Calif.) and were propagated in the above medium with the addition of 5-bromo-deoxyuridine (BUdR) at 25 ug/ml.

Human diploid cell (MRC-5) was obtained from the American Type Culture Collection (ATCC No. CCL171) and was propagated in the same medium used for BSC-40.

Vaccinia virus (strain WR, ATCC No. VR-119) was obtained from R. Condit and was grown in BSC-40 cells in DMEM + 5% gamma globulin free calf serum + 100 units/ml each penicillin and streptomycin. TK^- recombinants were selected on 143 TK^- cells in the same medium containing 25 ug/ml of BUdR and plaque-purified on the same cell line in DMEM containing 1% Noble agar (DIFCO, Detroit, MICH), 5% gamma globulin free calf serum, 100 units/ml each of penicillin and streptomycin and 25 ug/ml BUdR. Dilutions of virus stocks were made in phosphate-buffered saline (PBS, per liter: NaCl, 8 gm; KCl, 0.2 gm; NaH_2PO_4, 1.5 gm; K_2HPO_4, 0.2 gm) supplemented with 1 mM $MgCl_2$ and 0.01% bovine serum albumin (PBSAM).

The New York City Board of Health strain of vaccinia virus was purified from a commercial preparation of smallpox vaccine (Dryvax® Lot 321501G) marketed by Wyeth Laboratories (Marietta, PA). Smallpox vaccine was diluted with PBSAM (see below) and plaque-purified three times successively on BSC-40 cells. A stock (hereafter designated as v-NY) was prepared on BSC-40 cells from such a plaque-purified isolate and was used to construct recombinant viruses. Stocks of recombinant virus derived from v-NY were propagated in MRT-5000 cells.

6.1.2. PREPARATION, RESTRICTION AND MODIFICATIONS OF DNA

Unless specified otherwise, all methods used for the following procedures were as described in the indicated pages of Maniatis et al., 1982, Molecular Cloning, Cold Spring Harbor Laboratory: preparation of plasmid DNA (pp. 86-96), restriction digestion of DNA (pp. 98-106) and purification of restriction fragments from low-melting temperature agarose gels (pp. 157-161 and p. 170), reaction conditions for the Klenow fragment E. coli DNA polymerase enzyme (pp. 107-114), calf intestinal alkaline phosphatase (pp. 133-134) and ligase reactions (p. 146), preparation of nick translated probes (pp. 109-112) and procedures for DNA-DNA hybridization (pp. 324-325).

6.2. CONSTRUCTION OF PLASMID VECTORS CONTAINING VACCINIA VIRUS PROMOTER LIGATED TO THE CODING SEQUENCES OF LAV/HTLV III ENV GENE

The following subsections describe the construction of various plasmid vectors containing coding sequences of the LAV/HTLV III envelope gene preceded by vaccinia virus transcriptional control sequences; these chimeric sequences are flanked by TK DNA. These recombinant plasmid vectors were later used to insert the LAV/HTLV III envelope coding

sequences into the genome of vaccinia virus through *in vivo* recombination.

In the subsections below, the LAV/HTLV III envelope coding sequence was purified from pRS-3, a subclone of lambda J19 (Wain-Hobson, S., et al., 1985, Cell 40:9), and inserted into plasmid pGS20 (Mackett, M., Smith, G.L. and Moss, B., 1984, J. Virol. 49, 857-864) downstream with respect to the vaccinia 7.5K promoter contained in pGS20, in order to construct pv-env1, pv-env2, pv-env5 and pv-env7. In each of these constructions, the chimeric gene (*i.e.* the 7.5K vaccinia promoter ligated to the LAV/HTLV III specific nucleotide sequence) is flanked by vaccinia TK gene sequences.

As previously explained, pRS-3 consists of a 3,840 base pair *Eco*RI to *Sst*I fragment of the LAV/HTLV III DNA insert contained in lambda J19 cloned into the *Eco*RI and *Sst*I site of pUC18. It was obtained by cloning the *Sst*I restriction fragment of J19 into the *Sst*I site of pUC18 to create pBT-1 which was then digested with *Eco*RI and re-ligated. This DNA was used to transform *E. coli* and plasmid pRS-3 was isolated. The LAV/HTLV III specific DNA contained in pRS-3 is from the *Eco*RI site located at nucleotide 5289 to the *Sst*I site located at nucleotide 9129 on the LAV/HTLV III genome (Wain-Hobson, S., et al., 1985, Cell 40:9); see FIG. 2.

6.2.1. CONSTRUCTION OF PLASMID VECTORS CONTAINING VACCINIA VIRUS PROMOTER LIGATED TO THE 3' CODING SEQUENCES OF LAV/HTLV III ENV GENE

Five micrograms of pRS-3 plasmid DNA was digested to completion with restriction enzyme *Kpn*I and the resulting fragments were resolved on a 1% low-melting temperature agarose gel. A 2.68 kbp (kilobase pair) fragment was isolated and purified. This fragment contained LAV/HTLV III-specific DNA from nucleotide number 5889 to 8572

including sequences (5889-8349) that encode the C-terminal portion of the LAV/HTLV III envelope protein.

One microgram of this fragment was mixed with 0.5 ug of pGS20 DNA which had been previously linearized with restriction enzyme BamHI. Ligation of these two fragments was allowed to proceed for 24 hours at 4°C in the presence of oligodeoxynucleotide linkers consisting of 0.6 ug of 5'-GATCCACCATGGTAC-3'-OH and 0.3 ug of 5'-CATGGTG-3'-OH. These linkers served (a) to convert the KpnI cohesive ends of the 2.68 kbp fragment derived from pRS-3 plasmid DNA to BamHI cohesive ends which are complementary to the BamHI cohesive ends of the cleaved pGS20 DNA and (b) to provide a translation initiation sequence (ATG) in the correct reading frame with respect to the LAV/HTLV III envelope gene sequence as well as nucleotide sequences required for efficient translation of the transcribed mRNA. The ligation mixture was used to transform E. coli strain MC1000. Plasmid DNA from ampicillin resistant transformants was tested for the orientation of the insert and the regeneration of BamHI, NcoI and KpnI sites at the ligation junctions. The confirmed structure of the desired plasmid, pv-env2, is shown in FIG. 3 in which the carboxy coding portion of the LAV/HTLV III envelope gene, corresponding to nucleotide numbers 5889-8572 (as shown in FIG. 2), is located downstream with respect to the 7.5K vaccinia promoter. The LAV/HTLV III envelope sequence is positioned in the correct reading frame with respect to the initiation ATG supplied by the linker DNA.

6.2.2. CONSTRUCTION OF PLASMID VECTORS CONTAINING VACCINIA VIRUS PROMOTER LIGATED TO THE 5' CODING SEQUENCE OF LAV/HTLV III ENV GENE

Five micrograms of pRS-3 plasmid DNA was digested to completion with AvaII restriction enzyme and the resulting fragments were resolved on a 1% low-melting temperature

agarose gel. A 0.82 kbp fragment was isolated and purified. This fragment contained LAV/HTLV III-specific sequences from nucleotide number 5671 to 6490 (as shown in FIG. 2). This includes sequences (nucleotide numbers 5766-6490) encoding the N-terminal portion of the envelope protein and 95 base pairs of 5′ proximal untranslated sequences of LAV/HTLV III. This fragment was treated with the Klenow fragment E. coli DNA polymerase in the presence of excess deoxyribonucleotide triphosphates. The resulting blunt-ended fragment was ligated to 0.5 ug of pGS20 DNA which was previously linearized with SmaI and treated with calf-intestinal alkaline phosphatase (CIAP). Ligation was allowed to proceed for 16 hours at 12°C. The ligation mixture was used to transform E. coli strain MC1000. Plasmid DNA from ampicillin resistant transformants was tested for the orientation of the LAV/HTLV III insert with respect to the vaccinia virus transcriptional control sequences. The confirmed structure for the desired plasmid, pv-env1, is shown in FIG. 4 in which the amino coding portion of the LAV/HTLV III envelope gene containing its own initiation ATG, corresponding to nucleotide numbers 5671 to 6490 (as shown in FIG. 2), is located downstream with respect to the vaccinia 7.5K promoter.

6.2.3. CONSTRUCTION OF PLASMID VECTORS CONTAINING VACCINIA VIRUS PROMOTER LIGATED TO THE ENTIRE CODING SEQUENCES OF LAV/HTLV III ENV GENE

Two micrograms of pv-env1 plasmid DNA was digested to completion by restriction enzymes StuI, PvuI, and XhoI. The resulting fragments were resolved on a 1% low-melting temperature agarose gel. Cleavage of pv-env1 with StuI and PvuI results in two 4 kbp fragments, one containing the 5′ portion of the LAV/HTLV III and the other containing pGS20; XhoI cleaves the 4 kbp fragment containing pGS20 into smaller fragments which, thus, enables the identification of the 4

kbp StuI/PvuI fragment containing the vaccinia virus transcriptional control element and the 5' portion of the LAV/HTLV III envelope coding sequences. This fragment was isolated, purified and ligated to a 6.5 kbp fragment generated by StuI and PvuI restriction digests of pv-env2. The ligation mixture was used to transform E. coli strain MC1000. Ampicillin-resistant transformants were selected. Plasmid DNA from individual transformants was tested for the regeneration of StuI and PvuI restriction sites and the presence of the parental 4 kbp and 6.5 kbp fragments. The desired plasmid, pv-env5, depicted in FIG. 4, contains the entire envelope gene of LAV/HTLV III corresponding to nucleotide numbers 5766 to 8349 (as shown in FIG. 2), ligated downstream from the vaccinia virus transcriptional control elements.

6.2.4 CONSTRUCTION OF PLASMID VECTORS CONTAINING VACCINIA VIRUS PROMOTER LIGATED TO THE LAV/HTLV III ENV GENE LACKING THE TRANSMEMBRANE (ANCHOR) SEQUENCE

Plasmid pv-env5 DNA (10 ug) was digested to completion with HindIII and the resulting fragment resolved on a 1% low melting point (LMP) agarose gel. The 3 kbp pair fragment containing the vaccinia 7.5K promoter and the 5' promoter of LAV/HTLV III env-coding sequences was purified and then treated with Klenow enzyme to fill in the cohesive ends. This fragment was then ligated to a multiple site cloning vector, p26, previously digested with EcoRI and then treated consecutively with Klenow enzyme and calf-intestinal alkaline phosphatase (CIAP). Ligation of the filled-in HindIII site in pv-env5 and the filled-in EcoRI site in plasmid p26 created a stop codon in phase with the coding sequence for env. This intermediate construct was designated as pv-env5/26.

Plasmid pv-env5/26 DNA was used to transform E. coli MC1000, amplified and purified. It was then digested with

BamHI and HpaI and the resulting fragments resolved on a 1% agarose gel. The 2.1 kbp pair fragment containing the LAV/HTLV III envelope coding sequence was isolated and then ligated to plasmid pGS20 previously digested with BamHI and SmaI. The resulting plasmid pv-env7 contains the vaccinia virus 7.5K promoter ligated to LAV/HTLV III-specific sequence starting from 96 base pairs upstream of the env initiation codon to the HindIII site at nucleotide number 7698 (Fig. 5). This plasmid therefore is lacking the presumed anchor sequence of the LAV/HTLV III env gene.

6.3 CONSTRUCTION AND CHARACTERIZATION OF RECOMBINANT VACCINIA VIRUS CONTAINING CHIMERIC LAV/HTLV III ENV GENE

Two parental strains of vaccinia virus were used for the construction of recombinant viruses: WR, the standard research strain, and v-NY, a derivative of the New York City Board of Health strain (see 6.1.1.).

Insertion of the chimeric LAV/HTLV III env sequences into the vaccinia virus genome was achieved by *in vivo* recombination, made possible by the fact that the chimeric genes in plasmids pv-env2, pv-env5 and pv-env7 are flanked by vaccinia virus sequences coding for the thymidine kinase (TK) gene. Introduction of these plasmids into cells infected with vaccinia virus allowed recombination to occur between the TK sequence on the plasmid and the homologous sequence in the vaccinia viral genome. Insertion of the chimeric gene occurs as the result of double recombinations in the flanking sequences. Such recombinants will have the chimeric gene inserted in the vaccinia TK gene and, consequently, will be phenotypically TK^-. These TK^- recombinants can be selected for growth in medium supplemented with BUdR which is lethal to TK^+ cells but not to TK^- cells. The general principle of this procedure has been described (Mackett, M., Smith, G.L. and Moss, B., 1984, J. Virol. 49, 857-864).

6.3.1. CONSTRUCTION OF RECOMBINANT VACCINIA VIRUS CONTAINING CHIMERIC LAV/HTLV III ENV GENE

A 100 mm dish of 80% confluent African Green Monkey Kidney Cells (strain BSC-40) were infected with vaccinia virus (strain WR) at an multiplicity of infection (moi) of 0.05. After 2 to 4 hours of incubation at 37°C, infected cells were overlaid with calcium phosphate coprecipitates of plasmid pv-env2 or pv-env5 DNA. The precipitates were prepared by adding 0.5 ml of 2XDNA-$CaCl_2$ solution dropwise to 0.5 ml of 2XHeBS solutions (2XDNA-$CaCl_2$ solution contains 20 ug of plasmid DNA in 0.5 ml of 0.25 M $CaCl_2$; 2XHeBS contains, per ml, 16 mg of NaCl, 0.74 mg of KCl, 0.25 mg of Na_2HPO_4 $2H_2O$, 2 mg of dextrose, and 10 mg of HEPES, at pH 7.08). DNA-calcium phosphate co-precipitates were allowed to form at room temperature for 30 minutes. Four hours after the overlay of precipitates, cells were washed once with 1XHeBs, incubated at 37°C for 3 minutes in the presence of 2 ml of 15% glycerol in 1XHeBs, and then washed once more with 1XHeBS and incubated with 10 ml of growth medium (DMEM + 10% fetal calf serum + 100 units/ml each penicillin and streptomycin). Two days later, infected cells were harvested and collected by centrifugation (4°C, 10 minutes at 2000 x g). Virus stocks were prepared by resuspending these cells in 1 ml of PBSAM, followed by two cycles of freezing and thawing and three 15 second sonications.

Recombinants were selected by plating 0.1 ml of 10^{-3} dilution of the viral stocks from above on 60 mm dishes of confluent human 143 TK^- cells, overlaid with 5 ml/dish of 1% Nobel agar (Difco, Detroit, MICH) 5% calf serum, 25 ug/ml BUdR in DMEM. Two days after plating, cells were stained by overlaying 2 ml/dish of agar-medium containing the same ingredients as above, plus 0.01% neutral red. Individual plaques were picked one day after staining, resuspended in 0.5 ml PBSAM, and aliquots (0.25 ml) of virus suspensions

were used to infect confluent 143 TK$^-$ cells seeded in 16 mm diameter wells under selective medium (DMEM + 10% fetal calf serum + 100 units/ml each streptomycin and penicillin + 25 ug/ml BUdR. Infected cells were collected by centrifugation and resuspended in 100 ul of PBS containing 0.5 mg/ml of trypsin and 0.2 mg/ml of EDTA. Cells were lysed by incubation at 37°C for 30 minutes, followed by 3 cycles of sonication, 20 seconds each. Cell lysates were collected on nitrocellulose filters by use of a multi-well filtering manifold (Schleicher and Schuell, Arlington, MA). The presence of LAV/HTLV III env specific DNA sequences in these samples was determined by DNA-DNA hybridization as described (Mackett, M., Smith, G.L. and Moss, B., 1982, Proc. Natl. Acad. Sci. 79, 7415-7419). ^{32}P-labeled plasmid pRS-3 DNA prepared by nick-translation was used as a hybridization probe. Recombinants that gave positive hybridization to this probe were further plaque-purified twice on 143 TK$^-$ cells under selective conditions (medium containing BUdR) and once on BSC-40 cells under non-selective conditions. After final confirmation by DNA-DNA hybridization, virus stocks were prepared from the thrice purified plaques on BSC-40 cells and used for subsequent characterization.

A schematic representation of the construction of the recombinant viruses is shown in FIG. 6 in which the LAV/HTLV III envelope gene sequence (env) located downstream from the vaccinia 7.5K promoter (p) is flanked by TK DNA sequences within the vaccinia genome. Virus stocks derived from recombination between vaccinia virus genome and plasmid pv-env5 were designated v-env5 and contain the entire LAV/HTLV III envelope gene. In the particular embodiment described in the examples herein, v-env5 contains the entire envelope gene as well as 96 base pairs of the 5'-proximal and 223 base pairs of the 3'-proximal untranslated sequences. Virus stocks derived from pv-env2 were designated v-env2 and contain most of the LAV/HTLV III envelope gene (<u>i.e.</u> the <u>KpnI</u>

fragment) but lack that part of the sequence which encodes the first 42 amino acids of the LAV/HTLV III envelope protein. Since the presumed signal sequence of the LAV/HTLV III envelope protein is located within the first 49 amino acids of the protein, v-env2 should produce a protein that lacks the signal sequence; therefore, one would expect that the LAV/HTLV III related protein produced by this recombinant virus will not be transported to the membrane. By contrast, v-env5, which contains the complete LAV/HTLV III env sequence, should produce a protein that is transported to the membrane. Virus stocks derived from pv-env7 are designated v-env7 and contain the complete N-terminus of the LAV/HTLV III envelope gene, but lack the sequence downstream of the HindIII site. Since the presumed transmembrane (anchor) sequence is missing in this construct, one would expect the LAV/HTLV III related protein produced by this recombinant to be secreted into the growth medium. This property is advantageous for the purification of this protein for its use as a diagnostic reagent or for its formulation as a subunit vaccine.

6.3.2. RESTRICTION PATTERNS OF VACCINIA-LAV/HTLV III ENVELOPE RECOMBINANTS

DNA from parental vaccinia virus strains WR and v-NY, as well as their derivatives v-env5 and v-env5NY, respectively, was isolated as previously described (Esposito et al., 1981, J. Virol. Methods, 2:175-180). They were digested with restriction enzyme HindIII and the resulting fragments were resolved on a 0.7% agarose gel. DNA from parental strain WR showed the typical restriction pattern (Fig. 2A) as previously described (DeFilippes, 1982, J. Virol. 43:136-149). The restriction pattern for v-NY was similar, but distinct from that of WR. The most obvious differences were in the length of HindIII B and C fragments, which are terminal fragments on the vaccinia virus genome.

Recombinants v-env5 and v-env5NY do not have the HindIII J fragment of their parental strains. Instead, two novel fragments, 5.4 kbp and 3.2 kbp in size, were observed. This observation is consistent with the result of the double recombination event that inserted the LAV/HTLV III envelope coding sequence into the vaccinia TK gene, which is located on the HindIII J fragment. Two fragments were generated by the insertion because of the presence of a HindIII site in the LAV/HTLV III sequence. This result also confirms the orientation of the LAV/HTLV III insert with respect to the vaccinia virus TK gene.

6.3.3. SOUTHERN BLOT ANALYSIS OF VACCINIA -LAV/HTLV III ENVELOPE RECOMBINANTS

The identity of the two novel HindIII fragments in the restriction digests of recombinant viral genomes was confirmed by Southern blot analysis. DNA fragments resolved on an agarose gel like the one presented in FIG. 7A were transferred to nitrocellulose filters, and were hybridized to a nick-translated probe specific for LAV/HTLV III envelope sequences. As shown in FIG. 7B, hybridization was detected only in the 5.4. and 3.2. kbp fragments. This result confirms the genomic structure of the recombinant virus as represented in FIG. 6.

6.4. EXPRESSION OF LAV/HTLV III ENVELOPE RELATED PROTEINS IN TISSUE CULTURE CELLS INFECTED BY RECOMBINANT VACCINIA VIRUSES

The recombinant vaccinia viruses carrying the chimeric LAV/HTLV III env genes were shown to be capable of expressing LAV/HTLV III envelope related proteins upon infection of cells in tissue culture. These proteins were also found to be immunoreactive with serum from AIDS patients.

6.4.1. IDENTIFICATION OF LAV/HTLV III ENVELOPE RELATED PROTEINS EXPRESSED IN CELLS INFECTED WITH RECOMBINANT VACCINIA VIRUS USING IMMUNOBLOTTING TECHNIQUES

A 100 mm dish of confluent BSC-40 cells was infected at a moi of 10 by wild-type vaccinia virus or with its recombinant derivatives, v-env5 or v-env2. Infection was allowed to proceed for 12 hours, at which time the cells were harvested, washed once with PBS, and collected by centrifugation. Infected cell pellets were resuspended in 1 ml of Laemmli sample buffer (Laemmli, U.K., 1970, Nature 227:680) and lysed by boiling for 4 minutes. Total cellular protein in a 75 ul aliquot of cell lysate was resolved by electrophoresis on a 7-15% gradient SDS-polyacrylamide gel. A sample of purified LAV/HTLV III virion and an aliquot of mock infected cell lysate were included as controls. The contents of the gel were electro-transferred to a sheet of nitrocellulose filter. The filter was first incubated in 5 ml PBS + 5% non-fat dry milk for 30 minutes at room temperature and then for 2 hours at room temperature in PBS + 5% non-fat dry milk + human serum from AIDS patients (1:100 dilution of heat inactivated serum). The filter was then washed 5 times with PBS + 0.05% Tween 20 (polyoxyethylene sorbitan monolaurate) and once with PBS alone. The washed filter was incubated for 2 hours at room temperature with 5 ml of PBS containing 1% normal goat serum (heat inactivated) plus a 1:3000 dilution of goat anti-human IgG-horseradish peroxidase conjugate. The same filter was again washed 5 times with PBS + 0.05% Tween 20, and once with TBS (0.5 M NaCl + 20 mM Tris-HCl, pH 7.5). Horseradish peroxidase conjugate bound on the filter was detected by reacting chloro-naphthol coloring reagents with the filter for 10 minutes at room temperature in the dark (chloro-naphthol coloring reagent was prepared by mixing solutions A and B just prior to use. Solution A: 20 ml cold 30% methanol + 60 mg 4 chloro-1-naphthol; solution B: 60 ul cold 30% hydrogen

peroxide + 100 ml TBS). Proteins bound on the filter that reacted with antibodies in AIDS patient serum would be detected by the coloring reagent through its binding to the goat anti-human IgG-horseradish peroxidase conjugates.

Results of this analysis, as shown in FIG. 8A, indicated that vaccinia virus recombinant v-env5 produced a family of three proteins that were immunoreactive specifically with serum from AIDS patients. These proteins had similar electrophoretic mobilities to the authentic LAV/HTLV III glycoproteins gp150, gp110, and gp41, which are believed to be encoded by the env gene (Robey, W.G. et al., 1985, Science 228: 593-595). These proteins were not produced in mock infected or wild-type vaccinia virus infected cells. Recombinant v-env2, which lacks the 5′ proximal sequences that code for the presumed initiating methionine and the first 42 amino acids of LAV/HTLV III envelope proteins, produced a protein of truncated size, but still immunoreactive with AIDS patient serum. Presumably, translation of the env sequence in recombinant v-env2 initiates from the AUG codon transcribed from the linker sequence used in the construction of this recombinant (see Section 6.2.1.).

In a second experiment, two cell lines (BSC-40 and Hela) were infected with v-env5 and v-env2. The LAV/HTLV III specific proteins in the infected cell lysates were assayed by Western immunoblots as described below.

Confluent monolayers of BSC-40 or Hela cells were infected with recombinant viruses v-env5 or v-env2 at a multiplicity of infection (moi) of 50 plaque-forming units (pfu) per cell. Twelve hours after infection cells were washed twice in phosphate-buffered saline, resuspended in Laemmli sample buffer and boiled for 5 minutes. Proteins from infected cells were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), electrotransferred to nitrocellulose membrane, and reacted

with pooled serum from LAV/HTLV III/HTLV-III seropositive individuals. Immunoreactive proteins were detected by protein A, which was labeled with ^{125}I (Amersham, MA) by the chloramine-T method (following manufacturer's instructions). In FIG. 8B, lanes 1 and 5 contain proteins from mock-infected cells; lanes 2 and 6, from cells infected with wild-type vaccinia virus; lanes 3 and 7, from v-env5 infected cells; and lanes 4 and 8, from v-env2 infected cells. Sucrose-gradient purified LAV/HTLV III virion proteins were used as control (LAV/HTLV III) and the positions of envelope proteins gp150, gp110 and gp41 were as indicated. Molecular weight standards were expressed in kilodaltons (kD).

Three major proteins immunoreactive with pooled serum from seropositive individuals were detected in the v-env5 infected cells (FIG. 8B, lane 3 and 7). The molecular weights of these proteins were estimated to be 150 kD, 120 kD and 41 kD, similar to those of LAV/HTLV III envelope glycoproteins gp150, gp110 and gp41.

Recombinant virus v-env2 lacked the putative signal sequence for LAV/HTLV III env, but was able to produce at least three immunoreactive polypeptides of molecular weights 99 kD, 68 kD and 40 kD (FIG. 8B, lanes 4 and 8).

6.4.2. IDENTIFICATION OF LAV/HTLV III ENVELOPE RELATED PROTEINS EXPRESSED IN CELLS INFECTED WITH RECOMBINANT VACCINIA VIRUS USING IMMUNOPRECIPITATION TECHNIQUES

The immunoprecipitation assay described below demonstrated that cells infected with the recombinant vaccinia viruses of the present invention synthesize proteins that are immunoreactive specifically with serum from AIDS patients.

A 100 mm dish of confluent BSC-40 cells was infected by wild-type vaccinia virus, or its recombinants v-env5 or v-env2, at a moi of 10. At 9.5 hours post infection, growth medium was replaced by methionine-free DMEM with no serum

supplements. At 10 hours post infection, the media was replaced by 2 ml of methionine-free DMEM containing 100 uCi/ml of [^{35}S]-methionine and labeling was allowed to proceed for 2 hours at 37°C. At the end of the labeling period, cells were washed once with PBS and collected by centrifugation. Cell pellets were resuspended in 1 ml of lysis buffer: 1% NP-40 (polyoxyethylene (9) p-tert-octylphenol), 0.5% sodium deoxycholate, 0.1 M NaCl, 0.01 M Tris-HCl, pH 7.4, 1 mM EDTA and the lysate was cleared by centrifugation for 1 minute in an Eppendorf microcentrifuge.

Immunoprecipitation was carried out by adding 5 ul of heat inactivated human serum, either from a control population or from AIDS patients, to 100 ul of cell lysate. After a 1 hour incubation at 4°C, 60 ul of activated *Staphylococcus aureus* cells (Pansorbin cells, Calbiochem-Behring Corp., LaJolla, CA) was added and incubation was allowed to continue for another hour at 4°C. Immunoprecipitation complexes were collected by centrifugation for 30 seconds in an Eppendorf microcentrifuge at 4°C and washed once in 1 M NaCl + 0.1% NP-40 + 0.01 M Tris-HCl, pH 7.4 and twice in RIPA buffer [10 mM Tris-HCl, pH 7.2, 0.15 M NaCl, 1% sodium deoxycholate, 1% Triton X 100 (polyoxyethylene (9-10) p-tert-octylphenol), 0.1% sodium lauryl sulfate]. Washed immunoprecipitates were resuspended in 50 ul of Laemmli sample buffer, boiled for 1 minute and centrifuged for 1 minute in an Eppendorf microcentrifuge. Immunoprecipitated proteins present in the supernatant were analysed by electrophoresis on 15% SDS-polyacrylamide gels. After electrophoresis, the gels were stained with Coomassie blue dye, treated with sodium salicylate (30 minutes at room temperature in 1M sodium salicylate) and dried for fluorography.

Results of this analysis, as shown in FIG. 9A, indicated that recombinant v-env5 synthesized a family of proteins immunoreactive specifically with AIDS patient serum. These

proteins had apparent molecular weights of 160, 140, 120, 42 and 40 kilodaltons (kD), corresponding approximately with the apparent molecular weights of the envelope related glycoproteins reported for LAV/HTLV III (Robey, W.G. et al., 1985, Science 228: 593-595). These proteins were not present in mock infected or wild-type vaccinia virus infected cells, nor were they immunoprecipitated by control human serum. In v-env2 infected cells, a truncated protein of an apparent molecular weight of 95 kD was produced and recognized by AIDS patient serum. This truncated form of LAV/HTLV III env related protein is most likely initiated from the AUG codon encoded by the linker sequence located 5′ proximal to the LAV/HTLV III insert in this recombinant.

6.4.3. ^{3}H-GLUCOSAMINE LABELING OF LAV/HTLV III ENVELOPE RELATED PROTEINS PRODUCED BY VACCINIA-LAV/HTLV III RECOMBINANT VIRUSES

The radioimmunoprecipitation assay described below indicated that the vaccinia-LAV/HTLV III recombinant viruses of the present invention produced glycosylated envelope proteins.

Hela cells were either mock infected (see FIG. 9B lanes 1 and 5), or infected separately by wild-type vaccinia virus (lanes 2 and 6), recombinants v-env5 (lane 3 and 7) or v-env2 (lanes 4 and 8), all at an moi of 50 pfu/cell. ^{3}H-glucosamine (0.25 uCi at 23 mCi/mg, Amersham) was added to culture medium from 4 to 16 hours after infection. Cells were washed twice with phophate-buffered saline and lysed in buffer containing 0.1 M NaCl, 0.01 M Tris-HCl pH 7.4, 1 mM EDTA, 1% NP-40 (polyoxyethylene (9) p-tert-octylphenol) and 0.5% sodium deoxycholate. Aliquots of cell lysates were mixed with either normal human serum (lanes 1-4) or pooled serum from LAV/HTLV III seropositive individuals (lanes 5-8). Immunoreactive proteins were precipitated by fixed

Staphylococcus aureus cells bearing protein A, resolved by SDS-PAGE and detected by fluorography.

The results of the radioisotopic labeling with glucosamine as shown in FIG. 9B lane 7 indicated that the v-env5 recombinant-made proteins, like the envelope proteins of LAV/HTLV III, were also glycosylated. Differences in the glycosylation patterns could account for the slight variations observed in the electrophoretic mobilities of recombinant-made proteins as compared to LAV/HTLV III virion glycoproteins.

As would be expected from a protein lacking a signal peptide, no N-linked glycosylation with ^{3}H-glucosamine was observed in v-env2 infected cells (FIG. 9B, lane 8).

6.4.4. PULSE-CHASE IMMUNOPRECIPITATION ANALYSIS OF LAV/HTLV III ENVELOPE-RELATED PROTEINS PRODUCED BY VACCINIA-LAV/HTLV III RECOMBINANT VIRUSES

It has been suggested that gp150 of LAV/HTLV III is the precursor from which an exterior protein gp110 and a transmembrane protein gp41 are derived. The "pulse-chase" immunoprecipitation assay described below, indicates that the vaccinia-LAV/HTLV III recombinant-made 150 kD, 120 kD and 41 kD proteins have a precursor-product relationship similar to that suggested for gp150, gp110 and gp41 of authentic LAV/HTLV III.

Confluent monolayers of Hela cells were infected with wild-type vaccinia virus (see FIG. 9C, lanes 1-6), recombinant v-env5 (lanes 7-12), or v-env2 (lanes 13-18), all at a moi of 50 pfu/cell. At 10.5 hours post-infection, cells were labeled with ^{35}S-methionine (greater than 800 Ci/mmol, Amersham) at 100 uCi/ml for 15 minutes. At the end of the labeling period, cells were washed once with 2 ml of prewarmed chase-medium (Dulbecco modifed Eagle's medium + 3 mg/ml L-methionine + 5% calf serum + 100 units/ml penicillin and 100 ug/ml streptomycin) and re-fed with 1 ml of the same

medium before being returned to the incubator. At various times afterwards, cells were washed and lysed as previously described in Section 6.4.3. and proteins from cell lysates were immunoprecipitated with pooled serum from LAV/HTLV III seropositive individuals. Immunoprecipitated proteins were resolved by SDS-PAGE and detected by fluorography. The duration of each chase was as follows: 0 hours (lanes 1, 7, 13); 0.5 hours (lanes 2, 8, 14); 1 hour (lanes 3, 9, 15); 2 hours (lanes 4, 10, 16); 6 hours (lanes 5, 11, 17) and 12 hours (lanes 6, 12, 18). Results shown in FIG. 9C, lanes 7-12 indicated the same precursor-product relationship for the recombinant-made 150 kD, 120 kD and 41 kD proteins as the authentic LAV/HTLV III gp150, gp110 and gp41. The processing of the 150 kD protein appeared to be slow and inefficient in Hela cells, since by 6 hours after the pulse-labeling less than 50% of the radioactivity in the 150 kD protein was chased into 120 kD and 41 kD proteins. Preliminary results indicated that this processing is more efficient in certain types of human peripheral blood cells infected with the same recombinant virus. These experiments also indicated that the env sequence in v-env2 which lacked the putative signal sequence for LAV/HTLV III env was expressed as an unmodifed 87 kD precursor (780 amino acids), which was processed to a 99 kD intermediate and cleaved into the 68 kD and 40 kD polypeptides (FIG. 9C, lanes 13-18).

6.4.5. PRESENCE OF LAV/HTLV III ENVELOPE RELATED PROTEIN IN THE GROWTH MEDIUM OF CELLS INFECTED WITH VACCINIA-LAV/HTLV III RECOMBINANT VIRUSES

The radioimmunoprecipitation assay described below demonstrated that the immunoreactive proteins produced by the recombinant vaccinia virus of the invention can be expressed and processed in a pattern similar to that of the authentic LAV/HTLV III envelope glycoprotein.

Confluent monolayers of Hela cells were either mock infected (see Fig 9D, lane 1), or infected separately by wild-type vaccinia virus (lane 2), recombinants v-env5 (lane 3) or v-env2 (lane 4), all at an moi of 20 pfu/cell. Cells were labeled with ^{35}S-methionine (greater than 800 Ci/mmole, Amersham) at 100 uCi/ml from 10 to 12 hours after infection. At the end of the labeling period, medium was removed and clarified by centrifugation for 2 minutes at 12,000 x g before use for immunoprecipitation with pooled serum from LAV/HTLV III seropositive individuals. Proteins from infected cells immunoprecipitated by the same serum are shown in the panel labeled "Pellet" and those from the medium are shown in the panel labeled "Supe".

As would have been expected for gp110 of LAV/HTLV III, the recombinant-made 120 kD protein was also detected in infected cell medium (FIG. 9D, lane 3). These results demonstrated that the recombinant virus v-env5 was able to express the LAV/HTLV III env gene and to produce immunoreactive proteins that were processed in a pattern similar to authentic LAV/HTLV III envelope glycoproteins.

As would have been expected for proteins lacking signal peptides, no LAV/HTLV III envelope related polypeptide was detected in the v-env2 infected cell medium (FIG. 9D, lane 4).

On the other hand, cells infected with v-env7 expressed a truncated protein of 130 kD. This protein is missing the C-terminal 217 amino acids containing the anchor sequences of the LAV/HTLV III envelope protein. This truncated protein (gp130) was secreted into the growh medium much more efficiently than gp110 or its precursor gp150 (FIG. 9E). At the same time, gp130 is not efficiently processed into gp110, even though the cleavage site is still present on the truncated molecule.

6.5. IMMUNOPOTENCY OF VACCINIA LAV/HTLV III ENV RECOMBINANT VIRUS

The recombinant viruses carrying chimeric LAV/HTLV III env gene were shown to be capable of eliciting antibody response against LAV/HTLV III in two strains of mice and one species of sub-human primate.

6.5.1. IMMUNOGENICITY OF VACCINIA-LAV HTLV III ENV RECOMBINANTS IN MICE

The immunogenicity of proteins expressed by recombinant vaccinia viruses v-env5 and v-env2 was examined. The experiments outlined below indicate the ability of these recombinant viruses to elicit an immune response to all major glycoproteins of LAV/HTLV III.

Two strains of mice, one inbred (C57Bl6J) and one outbred (ICR) were inoculated with recombinant viruses v-env2 or v-env5. All animals were 5-7 weeks old at the time of inoculation. Four routes of inoculation were used: footpad, tail scarification, intranasal and intraperitoneal. Footpad inoculation was done by injecting 25 ul (5 x 10^6 pfu) of recombinant viruses into each of the rear footpads. Tail scarification was done by roughing up the skin at the base of the tail with a bifurcated needle and applying 10 ul (2 x 10^7 pfu) of recombinant viruses onto the scarified surface. Intranasal inoculation was done by placing 10 ul (2 x 10^7 pfu) of recombinant viruses on the noses of the mice and allowing the mice to breathe in the inoculum. Intraperitoneal injections were done by injecting 10 ul (2 x 10^7 pfu) of recombinant viruses into the peritoneal cavity of the mice. All viral stocks and dilutions were made as described in section 6.1.1. Serum samples from individual mice were collected at two-week intervals after inoculation and analyzed by both enzyme linked immunosorbant assay (ELISA) and Western blot analysis as described below.

6.5.1.1. SEROCONVERSION OF MICE IMMUNIZED WITH VACCINIA-LAV/HTLV III ENV RECOMBINANTS AS DEMONSTRATED BY ELISA

ELISA data on the 6-week serum samples are summarized in Table I. Recombinants v-env2 and v-env5 sero-converted 100% and 95% of the C57B16J mice, respectively. For ICR mice, the rate of sero-conversion was 44% for v-env2 and 100% for v-env5. Total sero-conversion as well as the highest average ELISA titer was obtained in mice immunized by tail scarification.

TABLE I

Seroconversion of Mice Inoculated with Recombinant Viruses Carrying Chimeric LAV/HTLV III env Gene

Recombinant Virus	Route of Inoculation	Seroconversion of Mice Inoculated* ICR	C57B16J
v-env2	Footpad	3/3	4/5
	Tail Scarification	3/3	4/5
	Intranasal	0/3	4/5
	Intraperitoneal	Not done	3/5
v-env5	Footpad	4/4	5/5
	Tail Scarification	3/3	5/5
	Intranasal	3/3	4/5
	Intraperitoneal	Not done	5/5

* Seroconversion, determined by ELISA as described in the accompanying text, is expressed as the number of mice seroconverted over the total number of mice inoculated within each group.

The ELISA data was generated as follows: Purified, inactivated LAV/HTLV III virions were diluted in carbonate

buffer (50 nM sodium carbonate, pH 9.6) and added to 96-well microtiter plates (100 ul/well containing 0.2 ug of inactivated LAV/HTLV III). Binding was allowed to proceed at 4°C overnight. Unbound protein was aspirated and washed first with 200 ul/well of PBS + 5% non-fat dry milk and then with 300 ul/well of 4% sucrose solution. After excess sucrose solution was aspirated off, plates were allowed to dry at room temperature (1 hour). Then, 50 ul of PBS containing 2.5 ul of mouse serum samples was added to each well and allowed to react at 37°C for 1 hour. At the end of the incubation period, wells were washed 5 times with PBS + 0.05% Tween-20 (polyoxyethylene sorbitan monolaurate). Fifty ul of goat anti-mouse horseradish-peroxidase conjugates (1:5000 dilution in PBS + 0.05% Tween-20 was added to each well and allowed to react at 37°C for 45 minutes. After 5 washes with PBS + 0.05% Tween-20, 100 ul of citrate-O-phenylenediamine-peroxide substrate was added. The substrate was made as follows: 0.294 g sodium dihydrate + 0.537 g sodium phosphate dibasic crystal were dissolved in 10 ml H_2O and adjusted to pH 5.0 with HCl; 2 ul of 30% H_2O_2 and 4 mg of O-phenylene-diamine were added just before use. Plates were incubated 30 minutes in the dark at room temperature. The reaction was stopped by adding 100 ul of 1.3 N sulfuric acid to each well and the optical absorbance of the reaction mixture at 490 nM was measured.

Results in Table I are expressed as ratios of sero-positive mice to the total mice inoculated. Serum samples were collected 6 weeks after inoculation and analyzed by ELISA. Serum samples from 5 uninoculated mice were used as controls. The mean ELISA titers for the control group were 0.021 $\pm$ 0.005 and 0.017 $\pm$ 0.010, for ICR and C57Bl6J mice, respectively. Serum samples from immunized mice that gave ELISA titers more than three standard deviations higher than the control group were scored as positive.

6.5.1.2. <u>SEROCONVERTED MICE PRODUCE ANTIBODIES AGAINST AUTHENTIC LAV/HTLV III ENVELOPE GLYCOPROTEINS AS DEMONSTRATED BY WESTERN IMMUNOBLOT ASSAY</u>

To demonstrate that mice inoculated with the recombinant viruses produced antibodies against authentic LAV/HTLV III envelope glycoproteins, serum samples from immunized mice were analyzed by a Western immunoblot technique as follows: five to seven week old male inbred mice (C57Bl6J, Jackson Laboratory) were immunized by tail scarification, which consisted of applying a 10 ul inoculum containing 2 x 10^7 pfu of recombinant vaccinia virus to abrasions generated with a bifurcated needle at the base of the tail. Animals were bled from the retro-orbital sinus at 8 weeks post inoculation and serum maintained frozen until use. Aliquots of serum samples diluted 50-fold in phosphate-buffered saline plus 0.2% NP-40 and 3% non-fat dry milk were reacted with LAV/HTLV III virion proteins which had been resolved by SDS-PAGE and immobilized on nitrocellulose filters by electro-transfer. LAV/HTLV III proteins recognized by these sera were detected by goat anti-mouse immunoglobulin conjugated with alkaline phosphatase.

Results of the 8-week serum samples from C57Bl6J mice inoculated with the recombinant viruses by tail scarification are shown in FIG. 10. All animals immunized with the recombinant viruses produced antibodies that reacted with LAV/HTLV III envelope glycoprotein gp41. Serum from some of the animals immunized with v-env5 (FIG. 10, lane a) also recognized gp150 and gp 110, indicating the ability of this recombinant virus to elicite an immune response to all major glycoproteins of LAV/HTLV III.

6.5.2. <u>IMMUNOGENICITY OF VACCINIA-LAV/HTLV III RECOMBINANT V-ENV5 IN SUB-HUMAN PRIMATES</u>

Immunogenicity of vaccinia-LAV/HTLV III recombinant v-env5 was studied in two sub-human primate species: <u>Macaca</u>

fascicularies and chimpanzees. Results described in the following subsections demonstrated that v-env5 was capable of eliciting humoral and cell-mediated immunity in both species.

6.5.2.1. HUMORAL RESPONSE IN MACAQUE MONKEYS IMMUNIZED WITH V-ENV5

Nine long-tailed macaques (*Macaca fascicularis*), of juvenile to adult age, were used to study the immunogenicity of vaccinia-LAV/HTLV III recombinant v-env5. All animals were pre-screened for the absence of antibodies to vaccinia virus and LAV/HTLV III, and the absence of simian T-lymphotropic or simian AIDS viruses or antibodies to these viruses. Four monkeys were inoculated with 2×10^8 pfu of v-env5 and four with 2×10^7 pfu of the same virus. One animal was inoculated with 2×10^7 pfu of a control recombinant vaccinia virus (a vaccinia-herpes simplex gD1 recombinant v-HSgD1). All animals were inoculated by skin scarification. Serum and heparinized blood samples were collected prior to inoculation and at 4, 6 and 8 weeks post-inoculation. Ten weeks after the primary inoculation all animals were given a second inoculation of 2×10^8 pfu of the same virus. All animals showed self-healing skin lesions typical of vaccinia infections and normal physiological indicators throughout the course of the experiment. Humoral response was analysed by ELISA and Western blot assays.

For ELISA analysis, serum samples were diluted 50-fold in PBS containing 3% non-fat dry milk and 0.2% NP-40 (polyoxyethylene(9)*p*-*tert*-octylphenol), and were reacted with LAV/HTLV III virion proteins which had been immobilized on microtiter wells. The procedure for ELISA was identical to that described in Section 6.5.1.1, with the exception that the second antibody used was goat anti-human antibody conjugated to horseradish peroxidase.

The results shown in FIG. 11 demonstrate that, although only two animals showed seroconversion after primary

inoculation, all animals seroconverted after the second immunization.

To determine what antibodies were produced in the immunized animals, serum samples collected at 4 weeks after the second immunization were analyzed by Western blot. Two protocols were used for the optimal detection of the two envelope glycoproteins, gp41 and gp110. For optimal detection of gp41, serum was diluted 50 fold in PBS plus 3% non-pasteurized milk and 0.2% NP-40, and reacted at room temperature for 1 hour with purified LAV/HTLV III virion protein immobilized on a nitrocellulose filter. The filters were washed with PBS plus 0.2% NP-40 and reacted with alkaline phosphatase conjugated goat anti-human immunoglobulin antibody (Zymed, CA) at a 1-2000 dilution in PBS buffer plus 0.2% NP-40. For the detection of gp 110, serum was diluted 50 fold in PBS plus 3% non-pasteurized milk and at room temperature for 1 hour with purified LV/HTLV III virion protein immobilized on a nitrocellulose filter. The filters were washed in PBS plus 0.05% Tween-20 and then reacted with alkaline phosphatase conjugated goat anti-human immunoglobulin antibody (Zymed, CA) at a 1-2000 dilution in PBS buffer. In each procedure, the final washing after the second antibody reaction was done in 0.1 M Tris (pH 9.5) with 0.1 M NaCl and 5 mM $MgCl_2$. Antibody bound to antigens on the filter was detected by reacting the filter with a solution containing 0.1 M Tris (pH 9.5), 0.1 M NaCl, 5 mM $MgCl_2$, 0.33 mg/ml bromo-chloro-indolyl phosphate, and 0.17 mg/ml of nitro-blue-tetrazolium. After filters were reacted with the chromogens, they were rinsed in water, air-dried and photographed.

As shown in Fig. 12B, all animals that received two inoculations showed strong antibody reaction to gp41. In addition, four of these animals also produced antibodies which reacted strongly with gp110. Taken together, the results shown in FIG. 12A demonstrate that recombinant v-env5

is capable of eliciting the production of LAV/HTLV III envelope-specific antibodies in macaque monkeys.

6.5.2.2 CELL-MEDIATED IMMUNE RESPONSE IN MACAQUES IMMUNIZED WITH V-ENV5

Peripheral blood lymphocytes (PBL) were isolated from heparinized blood of macaques obtained 4 weeks following a second intradermal immunization with v-env5, or a control vaccinia recombinant virus expressing herpes simplex virus glycoprotein D (v-HSVgd1), by Ficoll-hypaque centrifugation. PBL were also isolated from macaque 81 which was vaccinated only once with v-env5, and from non-immunized macaques. The PBL were suspended in RPMI 1640 medium (GIBCO, Grand Island, N.Y.) supplemented with 10% heat-inactivated normal human serum, and 1×10^5 PBL, in a final volume of 0.1 ml medium, were placed into wells of round bottomed 96-well plates. To each well was added 0.1 ml medium containing ultraviolet (UV)-light inactivated v-env5 (1×10^6 pfu/ml prior to UV-inactivation), or non-disrupted LAV/HTLV III (1 mg/ml, approximately 1×10^5 $TCID_{50}$) that was purified by two cycles of sucrose gradient centrifugations from supernatants of LAV/HTLV III infected CEM cells, an HLA DR negative T cell leukemia line. Six days after stimulation, each well of PBL was labeled with 1 uCi ^{3}H-TdR (^{3}H-thymidine, New England Nuclear, Boston, MA) for 6 hours, the cells were harvested, and cpm ^{3}H-TdR incorporated was determined as the mean value of 4 replicate wells. The stimulation index was calculated by dividing the cpm ^{3}H-TdR incorporated into stimulated cells by the cpm incorporated into non-stimulated cells.

TABLE II

LAV/HTLV III INDUCED PROLIFERATIVE RESPONSES OF PBL FROM MACAQUES AFTER IMMUNIZATION WITH A VACCINIA RECOMBINANT VIRUS EXPRESSING LAV/HTLV III ENVELOPE GLYCOPROTEINS

		Virus Used For PBL Stimulation				
		None	LAV/HTLV III		V-env5	
Macaque No.	Immuni-zation	cpm*	cpm*	SI**	cpm*	SI**
67	v-env5	1,586	7,465	4.7	60,415	38.1
68	v-env5	2,245	9,075	4.0	28,638	12.8
74	v-env5	1,585	4,732	3.0	21,487	13.6
75	v-env5	581	8,645	14.9	37,847	14.1
76	v-env5	2,479	5,987	2.5	36,657	14.8
80	v-env5	1,077	13,922	12.9	24,752	23.0
81	v-env5	965	3,985	4.1	40,572	42.0
82	v-env5	2,581	8,580	3.3	46,847	18.1
73	V-HSVgD1	612	553	1.0-	56,217	91.9
26	none	2,911	1,822	1.0-	2,240	1.0-
27	none	1,228	1,072	1.0-	2,532	2.0

*cpm ^{3}H-TdR incorporated

** Stimulation Index.

As shown in Table II, all immunized macaques, including number 81, which received only one inoculation of v-env5, produced lymphocytes that proliferated in specific response to LAV/HTLV III stimulation *in vitro*. In contrast, lymphocytes from animal 73, which received vaccinia-herpes simplex recombinant v-HSVgD1 only responded to stimulation by vaccinia virus, but not by LAV/HTLV III. Control non-immunized animals did not produce lymphocytes that responded to either antigen. Furthermore, the response for lymphocytes

from macaques immunized with v-env5 was similar in magnitude to that from macaque 73, which was vaccinated with v-HSVgD1. These results indicated that the expression of the LAV/HTLV III env gene by the recombinant virus in macaque monkeys did not suppress T cell mediated immune responses <u>in vitro</u> or <u>in vivo</u>.

To determine what subset of lymphocytes from the immunized macaques was stimulated by LAV/HTLV III, we examined the stimulated PBL by two color immunofluorescence for expression of IL-2 receptors that are present on activated T cells and B cells. CD4 and CD8 antigens are present on T cells and CD20 (Bp35) antigen is present on B cells. The results shown in in Table III demonstrate that nearly all of the virus-stimulated PBL that express IL-2 receptors also express CD4 or CD8 antigens, whereas only 1.5% to 2% coexpress CD20 (Bp35) antigen. This result indicates that the stimulated lymphocytes are T cells.

TABLE III

EXPRESSION OF IL-2 RECEPTORS AND CD4, CD8 or CD20 (Bp35) ANTIGENS ON PBL FROM VACCINATED MACAQUES AFTER STIMULATION WITH LAV/HTLV III OR A RECOMBINANT VACCINIA VIRUS EXPRESSING LAV/HTLV III ENVELOPE GLYCOPROTEINS

Macaque Number	PBL Stimulated With	Total % IL-2R+ Cells	% IL-2R+ Cells Coexpressing		
			CD4	CD9	CD20(Bp35)
74	v-env5	27.7	48.6	52.2	2.0
80	v-env5	25.6	62.4	40.0	NT
74	LAV/HTLV III	14.7	50.0	59.0	1.9
80	LAV/HTLV III	12.0	58.0	38.0	1.5
80	no virus	0.3	0.3-	0.3-	0.3-

The results in Table III were obtained as follows: PBL were stained simultaneously with phycoerythrin-conjugated monoclonal antibody 2A3 (Becton-Dickinson, Inc., Mountain View, CA) to the interleukin-2 receptor (IL-2R), and fluorescein-conjugated monoclonal antibodies 1F5 (Genetic Systems Corp., Seattle, WA) to CD20, G10-1 (Genetic Systems Corp., Seattle, WA) to CD8, or $T4_a$ (Ortho Diagnostics, Raritan, N.J.) to CD4. The antibodies were used at saturating concentrations as previously determined by titrations on lymphocytes analyzed by flow microfluorometry with a modified FACS IV sorter (Becton-Dickinson, Mountain View, CA). Quantitative two color analysis was performed as previously detailed (Ledbetter, J.A., et al., 1984, Perspectives in Immunogenetics and Histocompatibility, 6 119-129). The forward and right angle scatter gates were set to include lymphoblasts and a substantial number of small

lymphocytes. The values shown are those for the total percent IL-2R+ cells and for the percent IL-2R+ cells coexpressing CD4, CD8 or CD20 (Bp35).

It is well established that T cells, following antigenic stimulation, produce lymphokines including IL-2 which can promote differentiation and/or proliferation of T and B cells and can activate natural killer cells that are capable of lysing cells infected with a variety of viruses including AIDS virus. We therefore asked whether T cells from the vaccinated macaques produce IL-2 following stimulation with LAV/HTLV III.

To this end two experiments were conducted. For Experiment 1, PBL were isolated from heparinized blood of macaques 4 weeks following a second intradermal immunization with v-env5 or v-HSVgD1 constructed with the WR strain of vaccinia virus. For Experiment 2, PBL were isolated from macaques 4 weeks after a primary intradermal immunization with 2 x 10^5 pfu of v-env5, v-HSVgD1, or a v-env5 recombinant virus constructed with the New York City Board of Health vaccine strain of virus (v-env5NY). The PBL were suspended in RPMI 1640 medium supplemented with 10% heat-inactivated normal human serum, and 2 x 10^5 PBL were placed into wells of round bottomed 96-well plates. UV-light inactivated v-env5 (1 x 10^6 pfu/ml prior to UV-light inactivation) or purified LAV/HTLV III (1 ug/ml) were then added to the wells. Two days after stimulation, supernatants were harvested from the replicate wells and tested for their ability to support proliferation of IL-2 dependent CTLL-2 cells (provided by Dr. S. Gillis, Immunex Corp., Seattle, WA) that were washed free of IL-2 for 24 hours prior to the assay. During the last 6 hours of incubation with the supernatants, the cells were labeled with ^{3}H-TdR, and the amount of ^{3}H-TdR incorporated into the cells was determined. The units of IL-2 activity present in the supernatants were calculated as described from standard curves obtained by testing the effect of recombinant

human IL-2 (provided by Dr. Gillis) on proliferation of CTLL-2 cells. Results are presented in Table IV.

TABLE IV

IL-2 PRODUCTION BY PBL FROM VACCINATED MACAQUES AFTER STIMULATION WITH LAV/HTLV III OR RECOMBINANT VACCINIA VIRUSES EXPRESSING AIDS VIRUS ENVELOPE GLYCOPROTEIN

		IL-2 (units/ml)		
		Supernatants of PBL Stimulated With		
Macaque No.	Immunization	No Virus	LAV/HTLV III	v-env5
Experiment 1:				
67	v-env5	0	28.0	96.0
68	v-env5	0	16.0	124.0
74	v-env5	0	9.0	52.0
73	v-HSVgD1	0	0	108.0
26	none	0	0	0
Experiment 2:				
03	v-env5	0	14.4	55.8
05	v-env5NY	0	16.8	33.3
49	v-env5NY	0	7.1	26.7
52	v-env5NY	0	2.4	27.6
59	v-HSVgD1	0	0	27.6
26	none	0	0	0
27	none	0	0	0

The results of Experiment 1 in Table IV show that supernatants from v-env5- or LAV/HTLV III-stimulated PBL, obtained from macaques immunized twice with v-env5, contained IL-2 as shown by their ability to induce proliferation of CTLL-2 cells, an IL-2-dependent cell line. Similarly, as shown in Experiment 2 of Table IV, IL-2 was detected in supernatants of LAV/HTLV III- or v-env5-stimulated PBL from

macaque 03, which was immunized once with v-env5, and from all three macaques that were immunized once with the "vaccine" strain of the same recombinant (v-env5NY, see section 6.3). In contrast, PBL from macaques 73 and 59, that were immunized with the vaccinia-HSV gD-1 recombinant virus, produced IL-2 following stimulation with v-env5 only, and not after stimulation with LAV/HTLV III. Non-immunized macaques 26 and 27 did not produce detectable IL-2 after stimulation with either LAV/HTLV III or the recombinant vaccinia virus. Since primarily T cells with helper/inducer activity produce IL-2 after antigenic stimulation, these results demonstrate the presence of helper T cells, which recognize LAV/HTLV III, in macaques immunized with the recombinant viruses. In addition to their probable role in the differentiation of B cells to produce antibodies to LAV/HTLV III envelope antigens, these IL-2 producing T cells may be involved in differentiation and/or expansion of effector cells, such as cytotoxic T lymphocytes or natural killer cells that can kill virus infected cells.

6.5.2.3 HUMORAL RESPONSES IN CHIMPANZEES IMMUNIZED WITH V-ENV5NY

Two chimpanzees of juvenile age were inoculated intradermally with 5 x 10^8 pfu of v-env5NY, the "vaccine" strain of v-env5. One animal was inoculated intradermally with the same titer of a vaccinia-herpes simplex gD recombinant, v-HSVgD1NY constructed from the same parental vaccinia strain (v-NY) as v-env5NY. All animals were given a second inoculation of the same dosage 8 weeks after the primary immunization. Serum samples were collected bi-weekly following immunization and were assayed for LAV/HTLV III-specific antibodies by ELISA and Western blot. All animals showed self healing skin lesions typical of vaccinia virus infection and had normal physiological indicators throughout the course of the experiment.

Methods for ELISA on chimpanzee sera were identical to those used for macaque sera. The results shown in Table V indicate that both experimental animals (124 and 149) had sero-converted by 8 weeks after primary immunization and that the antibody levels continued to rise after the second immunization. Control animal (134) remained seronegative.

TABLE V

ELISA OF SERUM SAMPLES FROM CHIMPANZEES IMMUNIZED WITH RECOMBINANT VACCINIA VIRUSES

Time of Sampling	ELISA Reading For LAV/HTLV III-Specific Serum Antibodies: Chimp No. 134 v-HSVgDINY	Chimp No. 124 v-env5NY	Chimp No. 149 v-env5NY
Prebleed	0.084 ± 0.015	0.100 ± 0.005	0.123 ± 0.025
8 Wks. After 1st immunization	0.085 ± 0.010	0.185 ± 0.020	0.403 ± 0.027
2 Wks. After 2nd immunization	0.075 ± 0.012	0.237 ± 0.017	0.523 ± 0.073

To demonstrate that the LAV/HTLV III-specific antibodies detected in the immunized animals were directed against envelope glycoproteins, we analyzed the same sera by Western blot. The procedure used was optimized for the detection of gp41 antibody, and was identical to that used for the analysis of macaque sera. The results shown in FIG. 13 demonstrate that the LAV/HTLV III-specific antibodies made in both vaccinated animals are indeed against envelope glycoproteins, and that the level of these antibodies increased after the second immunization.

6.5.2.4 CELL-MEDIATED IMMUNE RESPONSES IN CHIMPANZEES IMMUNIZED WITH v-env5NY

Peripheral blood lymphocytes (PBL) isolated from the same animals described in 6.5.2.3 were used for the demonstration of cell-mediated immune responses in these animals. PBL were isolated from heparinized blood by Ficoll-hypaque centrifugation 4 weeks following the initial intradermal inoculation with the recombinant vaccinia viruses. The PBL were seeded at 1 x 10^5 cells per well in 96-well plates, in RPMI 1640 medium supplemented with 10% heat inactivated normal human serum and penicillin/streptomycin. Non-disrupted LAV/HTLV III (5 ug/ml) or LAV/HTLV III envelope glycoproteins (1 ug/ml) isolated by lentil lectin chromatography from purified LAV/HTLV III, was then added to the wells. Six days later, ^{3}H-thymidine incorporated into the cells was determined by liquid scintillation counting.

As shown in Table VI, lymphocytes from both animals (124 and 149) immunized with v-env5NY proliferated in response to stimulation by either LAV/HTLV III virion or purified envelope proteins (env). In contrast, animal 134, which received vaccinia-herpes simplex recombinant v-HSgD1NY, did not produce lymphocytes that recognized LAV/HTLV III.

TABLE VI

CELL MEDIATED IMMUNE RESPONSE IN CHIMPANZEES VACCINATED WITH VACCINIA RECOMBINANTS

Chimpanzee No.	Immunization	^{3}H-Thymidine Incorporated Into PBL Stimulated With: No Antigen	LAV/HTLV III	env
124	v-env5NY	2482	37,132	26,370
149	v-env5NY	375	20,292	27,115
134	v-HSVgD1NY	367	1,987	367
64	none	452	567	222
72	none	737	2,417	905

PBL from all of the chimpanzees showed high levels of ^{3}H-thymidine incorporation (38,870 to 109,790 cpm) following stimulation with phytohemagglutinin (PHA, 2 ug/ml), and high levels of ^{3}H-thymidine incorporation (42,172 to 12,067 cpm) after stimulation with X-irradiated, pooled, xenogeneic human PBL. PBL from v-env5 immunized and also from the v-HSVgD1NY-immunized chimpanzees demonstrated strong proliferative responses to vaccinia virus, whereas PBL from the non-immunized chimpanzees did not proliferate in response to stimulation wtih vaccinia virus.

Taken together, the results shown in Tables I-VI and Figs. 11-13 indicate that (a) the recombinant vaccinia virus v-env5 and its counterpart in vaccine strain, v-env5NY, were capable of eliciting LAV/HTLV III-specific humoral and cell-mediated immune responses in two species of sub-human primates, macaques and chimpanzees, and (b) the recombinant viruses did not suppress T cell-mediated immune responses <u>in vitro</u> or <u>in vivo</u>.

6.6 REDUCED NEUROTOXICITY OF RECOMBINANTS CONSTRUCTED WITH THE V-NY STRAIN OF VACCINIA VIRUS

Aliquots of viruses, containing 5×10^7 pfu in 25-50 ul, were injected intracerebrally into groups of five mice (ICR strain). The mortality ratio was scored 10 days after inoculation.

TABLE VII

MORTALITY OF MICE INOCULATED INTRACEREBRALLY WITH VACCINIA RECOMBINANTS

Inoculation	Dead/Injected
v-NY	0/5
v-env5NY	0/5
v-env2NY	0/5
v-env7NY	0/5
Smallpox vaccine (Wyeth)	3/5
v-env5	5/5
v-env2	5/5
v-env7	5/5
Saline	0/5

The results shown in Table VII indicate that the plaque-purified, tissue culture-derived vaccinia virus (strain v-NY) and its derivatives v-env5NY, v-env2NY and v-env7NY have reduced neurotoxicity as compared to WR strain and its derivatives. Since the New York City Board of Health strain was used as smallpox vaccine, recombinants based on

this strain should be more suitable for the development of a vaccine for human use.

7. EXAMPLE: BACULOVIRUS GAG RECOMBINANTS

In the following examples, various plasmid vectors were constructed containing chimeric genes comprising LAV/HTLV III gag coding sequences located downstream with respect to the transcriptional control sequences of AcNPV.

These chimeric genes containing the AcNPV polyhedrin promoter and the LAV/HTLV III gag coding sequence were inserted into the genome of AcNPV through *in vivo* recombination. Such recombinant viruses were identified and purified, and viral stocks were prepared from infected tissue culture cells. Immunoreactive LAV/HTLV III gag related proteins were shown to be produced by the recombinant AcNPV *in vitro*. Cells infected with recombinant viruses were shown to exhibit positive immunofluorescence. Finally, lysates from cells infected with recombinant AcNPV were positive in an ELISA assay when tested with serum from AIDS patients. A detailed description of each step in this embodiment of the invention is presented in the subsections below.

7.1. GENERAL PROCEDURES

7.1.1. CELLS AND VIRUSES

Spodoptera frugiperda cells, clone Sf9, were obtained from the American Type Culture Collection (ATCC No. CRL 1711) and were propagated in Grace's Antheraea medium (Gibco, KC Biologicals) containing 3.3 gm/l yeastolate (Difco) and 3.3 gm/l lactalbumin hydrolysate (Difco), 10% fetal bovine serum and 0.06 gm/l penicillin and 0.1 gm/l streptomycin (TNM-FH medium). Cells were grown at 28°C. *Autographa californica* nuclear polyhedrosis virus was obtained from Dr. Lois Miller (Department of Genetics and Entomology, University of Georgia, Athens, GA 30602) and was plaque-purified on Sf9

cells containing 1% agarose and 0.8X TNM-FH. Dilutions of virus stocks were made in TNM-FH.

7.1.2. PREPARATION, RESTRICTION AND MODIFICATIONS OF DNA

Restriction enzymes were purchased from Bethesda Research Laboratories, Inc. and conditions for restriction digestions were as suggested by the manufacturer. The Klenow fragment of *E. coli* DNA polymerase was used at 200 units/ml in 50 mM Tris-HCl (pH 7.4), 6 mM $MgCl_2$, 10 mM 2-mercaptoethanol at 37°C for 30 minutes.

Viral DNA for transfection was prepared from non-occluded virus (NOV) stocks of wild-type virus using the following procedure of Miller, L.K., Miller D.W. and Safer, P.: The NOV particles were pelleted from the supernatants by centrifugation through a cushion of 25% sucrose, 5 mM NaCl and 10 mM EDTA at 90,000 x g for 1 hour at 5°C. The supernatant was removed and the virus pellet resuspended in 0.5 ml lysis buffer (10 mM Tris, pH 7.6, 10 mM EDTA, 0.25% SDS). After the virus pellet was resuspended, proteinase K was added to a final concentration of 500 ug/ml and incubated overnight (approximately 16 hours) at 37°C with occasional agitation. The DNA was extracted with an equal volume of phenol:-chloroform:isoamyl alcohol (25:24:1). The DNA was then precipitated at -20°C by the addition of 1/10 volume 3M sodium acetate pH 5 and 2 volumes cold ethanol. After pelleting, the DNA was washed with 70% ethanol, dried, and resuspended in TE (10 mM Tris, 1 mM EDTA, pH 7.5) before use for transfection or restriction enzyme analysis.

Plasmid DNA was prepared using the maxiprep procedure of Summers, M.D. and Smith, G.E. as follows: One ml of Luria Broth (LB) supplemented with an appropriate antibiotic was inoculated with a single colony of bacterial cells from a freshly prepared plate. After incubation at 37°C for 12 to 18 hours, the 1 ml culture was transferred to 200 ml LB plus antibiotics in a 500-1000 ml flask, and incubated in a

shaking incubator (37°C) overnight (12-18 hours). The 200 ml cultures were transferred to centrifuge bottles, and centrifuged at 2,500 rpm for 10 minutes. After removing the supernatant, each cell pellet was resuspended in 30 ml STET buffer (8% sucrose, 0.5% Triton X-100, 50 mM EDTA (pH 8.0), 10 mM Tris-Cl, pH 8.0) at room temperature and transferred to a 125 ml flask. Then 3 ml of 10 mg/ml lysozyme (prepared fresh in STET buffer) was added to each flask which was swirled to mix, and incubated for about 5 minutes at room temperature. Each flask was then gently swirled (about once every 5 seconds) directly over a flame until the cells began to coagulate and turn white. The moment bubbles formed, the flasks were transferred to a boiling water bath for 45 seconds, after which the flasks were cooled in an ice water bath for 2 minutes or longer. The viscous mixtures were slowly poured into 50 ml round bottom centrifuge tubes, and centrifuged for 15 minutes at 16,000 rpm using an SW 28 rotor (Beckman, CA) in a preparative centrifuge. The supernatant from each tube was carefully poured into a 50 ml disposable polypropylene centrifuge tube to which 1 volume of isopropanol or 2 volumes of ethanol were added and the DNA was precipitated at -80°C for 20 minutes (or -20°C for 2 hours or longer), and centrifuged at 2,500 x g for 15 minutes or more. After removal of the alcohol, 2.5 ml Extraction Buffer (per liter: 12.1 g Tris, 33.6 g Na_2EDTA $2H_2O$, 14.9 g KCl, pH 7.5) was added to the pellet, which was then vortexed to loosen the lysate from the bottom of the tube. 100 ul of 10 mg/ml of RNase A (dissolved in 0.1X TE and pretreated by boiling for 10 minutes) was added and incubated for 30 minutes at 37°C. Approximately 200 ug proteinase K was added to each tube and then incubated at 40-50°C for about 30 minutes, at which time 250 ul of 10% Sarkosyl was added and the incubation continued for 3 hours or longer. In order to prepare CsCl gradients, 80 ul of 10 mg/ml ethidium bromide per ml of DNA solution was added to each tube. Then 1.04 g

of CsCl per ml of DNA solution was added to each tube and gently vortexed to dissolve. After transfer to Quick-Seal™ tubes, the gradients were centrifuged to equilibrium at 55,000 rpm for 16 hours in a 70.1 Ti fixed angle rotor resulting in two well-separated visible bands of DNA in which the upper band comprises linear and nicked DNA whereas the lower band comprises covalently closed circular DNA. The lower band (covalently closed circular DNA) was collected and put into a 15 ml polypropylene centrifuge tube to which water was added to bring the volume in each tube to about 6 ml. The ethidium bromide was extracted from the DNA using an equal volume of isoamylalcohol, and the pink (upper) phase was removed and discarded. The extraction was repeated until the upper phase was colorless, and the bottom (DNA) phase clear and colorless. Approximately 5 ml of DNA solution should have been left in each tube (if not, water was added to a final volume of 5 ml) to which 2 volumes of absolute ethanol were added. The DNA was precipitated at -20°C overnight or -80°C for 10 minutes, and pelleted at 2,500 x g for 20 minutes. After removal of all the alcohol, 500 ul of 0.1X TE was added to each pellet, which was then resuspended at 65°C for 10 minutes or longer. The DNA can be stored at 4°C.

Plasmid DNA was also prepared as described in Maniatis et al., 1982, Molecular Cloning, Cold Spring Harbor Laboratory (pp. 8696). The DNA ligase was purchased from New England Biolabs and was used at 10,000 units/ml in 50 mM Tris-HCl (pH 7.8), 10 mM $MgCl_2$, 20 mM DTT, 1mM ATP. Analysis of DNA on agarose gels was as described in Maniatis et al., 1982, Molecular Cloning, Cold Spring Harbor Laboratory.

7.2 CONSTRUCTION OF PLASMID VECTORS CONTAINING AcNPV PROMOTER LIGATED TO CODING SEQUENCES OF LAV/HTLV III GAG GENE (pAc-gag1)

The following subsections describe the construction of plasmid vectors containing coding sequences of the LAV/HTLV III gag gene preceded by AcNPV transcriptional control sequences and followed by polyhedrin DNA. These recombinant plasmid vectors were later used to insert the LAV/HTLV III gag coding sequences into the genome of baculovirus through in vivo recombination.

In the subsections below, the LAV/HTLV III gag coding sequence (FIG. 14) was purified from pKS-5, a subclone of lambda J19 (Wain-Hobson, S., et al., 1985, Cell 40:9) and inserted into plasmid Ac610 downstream with respect to the baculovirus polyhedrin promoter contained in pAc610, in order to construct pAc-gag1 (FIG. 15). In this construction, the LAV/HTLV III specific nucleotide sequence is preceded by the polyhedrin promoter forming a chimeric gene followed by polyhedrin gene sequence.

As previously explained, pKS-5 consists of a 3,148 base pair SstI to KpnI fragment of the LAV/HTLV III DNA insert contained in lambda J19 cloned into the SstI and KpnI site of pUC18. It was obtained by cloning the SstI restriction fragment of J19 into the SstI site of pUC18 to create pBT-1 which was then digested with KpnI and religated. This DNA was used to transform E. coli and plasmid pKS-5 was isolated. The LAV/HTLV III specific DNA contained in pKS-5 is from the SstI site located at nucleotide 224 to the KpnI site located at nucleotide 3372 on the LAV/HTLV III genome (Wain-Hobson, S., et al., 1985, Cell 40:9; see also FIG. 14).

Ten micrograms of pKS-5 were digested to completion by HincII and SstI and the resulting fragments were resolved on a 1% low-melting temperature agarose gel. The 1818 base pair fragment (approximately 0.20 ug) was excised from the gel. This fragment contained LAV/HTLV III-specific sequences from

nucleotide number 224 to 2042 as shown in Figure 14. It contains the coding region for the entire gag gene.

Five micrograms of pAc610 was digested to completion with SmaI and SstI and fractionated on a 1% low-melting agarose gel; the DNA band (approximately 0.4 ug) was excised from the gel (FIG. 15).

Both gel slices were melted at 65°C for 10 minutes and 3 ul of the 1818 base pair LAV/HTLV III specific fragment (digested with HincII and SstI) were ligated to one microliter of pAc610 (digested with SmaI and SstI) in a total volume of 40 ul. Incubation was at 23°C (room temperature) for 16 hours The ligation mixture was then heated for 10 minutes at 65°C and used to transform E. coli strain HB101. Plasmid DNA from ampicillin resistant transformants was tested for the presence of the LAV/HTLV III insert. The structure of this plasmid, pAc-gag1, is shown in FIG. 15.

7.3. CONSTRUCTION OF RECOMBINANT BACULOVIRUS CONTAINING CHIMERIC LAV/HTLV III GAG GENE (Ac-gag1)

AcNPV was plaque-purified and propagated on Sf9 cells. Wild type AcNPV DNA was isolated and pAc-gag1 purified as previously described (see Section 7.1.2.).

Insertion of the chimeric LAV/HTLV III gag sequences into the AcNPV genome was achieved by in vivo recombination made possible by the fact that the gag sequences in pAc-gag1 are flanked by AcNPV sequences coding. Co-transfection of pAc-gag1 plasmid DNA with wild-type AcNPV DNA allowed recombination to occur between the polyhedrin sequences on the plasmid and the homologous sequences in the AcNPV genome. Insertion of the chimeric gene occurs as a result of double recombinations in the flanking sequences. Such recombinants will have the chimeric gene inserted in the AcNPV polyhedrin gene and consequently will be unable to produce occlusion bodies. The recombinant plaques can be selected by visual inspection for lack of occluded virus.

One microgram of AcNPV DNA, 5 micrograms of pAc-gag1, and 15 micrograms of calf thymus DNA were mixed and ethanol precipitated. The precipitate was washed with 70% ethanol, allowed to air dry under a tissue culture hood, and resuspended in 437 microliters of H_2O. $CaCl_2$ was added to 0.25 M (63 microliters of 2 M $CaCl_2$ to 437 microliters of DNA in H_2O). While bubbling air through 500 microliters of 2XHEBS, the DNA solution was added dropwise (one drop/4 seconds). 2XHEBS contains, per ml, 16 mg NaCl, 2 mg D-glucose, 0.75 mg KCl, 0.5 mg $Na_2HPO_4 \cdot 12H_2O$, 9.5 mg pH 7.1. The mixture was incubated at room temperature for 30 minutes and added to a 60 mm tissue culture dish containing 3 x 10^6 Sf9 cells in 2 ml of Grace's insect media containing 10% fetal bovine serum (FBS) and antibiotics (no yeastolate or lactalbumin hydrolysate). The DNA solution was added dropwise to the cells, and the dish was incubated for 4 hours at 28°C. The dish was rinsed with 4 ml of Grace's medium + FBS + antibiotics and incubated for 90 seconds with Grace's medium + FBS + antibiotics + 20% dimethylsulfoxide.

The plate was rinsed three times with complete medium and incubated at 28°C with 4 ml of complete medium. After 5 days, supernatants were collected and clarified at 1000 x g for 5 minutes. The virus stock was titered on Sf9 cells and non-occluded plaques were identified by visual inspection. Non-occluded plaques were picked and re-plaqued twice further. These plaques were expanded on Sf9 cells and virus stocks were prepared and used for subsequent characterization. A schematic representation of the construction of the recombinant is shown in FIG. 16.

7.4. EXPRESSION OF LAV/HTLV III GAG RELATED PROTEIN IN TISSUE CULTURE CELLS INFECTED BY RECOMBINANT BACULOVIRUSES

The recombinant AcNPV carrying the chimeric LAV/HTLV III gag gene was shown to be capable of expressing LAV/HTLV III

gag related RNA and proteins upon infection of cells in tissue culture. These proteins were also found to be immunoreactive with serum from AIDS patients.

7.4.1. IDENTIFICATION OF LAV/HTLV III GAG SPECIFIC RNA IN CELLS INFECTED WITH Ac-gag1

Ten 100 mm dishes of Sf9 cells (approximately 10^7 cells/dish) were infected by wild-type AcPNV or its recombinant, Ac-gag1 at a multiplicity of infection (moi) of 4. At 24 hours post infection cells were harvested and washed twice in 0.15 M NaCl, 0.01 M Tris-HCl, pH 7.2. Polyadenylated RNA was isolated as described (Purchio, A.F. and Fareed, G.C., 1979, J. Virol. 29:763-769). The RNA was fractionated on a 1% agarose-formaldehyde gel (Lehrach, H., et al., 1977, Biochemistry 16:4743-4251), transferred to a nylon filter (Hybond) and irradiated with UV light. The filter was hybridized to ^{32}P-labelled pKS-5 DNA in 0.9 M NaCl, 50 mM sodium phosphate (pH 7.0), 5 mM EDTA, 0.1% SDS, 4XDenhardts, 0.4 mg/ml tRNA, 0.25 mg/ml calf thymus DNA, 50% formamide for 16 hours at 42°C. Filters were washed four times in 0.1% SDS, 0.25XSSC for 30 minutes at 65°C and exposed to Cronex 4 X-ray film using Lightening Plus intensifying screens. FIG. 17 shows that a major band of 2.2 kilobase pairs is detected in recombinant AcNPV infected cells, but not in wild-type infected cells.

7.4.2. IDENTIFICATION OF LAV/HTLV III GAG RELATED PROTEINS EXPRESSED IN CELLS INFECTED WITH RECOMBINANT BACULOVIRUS USING IMMUNOPRECIPITATION TECHNIQUES

The immunoprecipitation assay described below demonstrates that cells infected with the recombinant baculovirus of the present invention synthesize proteins that are immunoreactive specifically with serum from AIDS patients.

Sf9 cells were seeded onto 60 mm dishes (5 x 10^6 cells/dish) and infected with wild-type AcNPV in Ac-gag1. At

24, 48 and 72 hours post infection, the media was replaced by methionine-free media and cells were incubated for 30 minutes at 28°C. After this time, the media was replaced by methionine-free media containing 100 uCi/ml [^{35}S] methionine and labeling was allowed to proceed for 2 hours at 28°C.

At the end of the labeling period, cells were washed twice with 0.01 M Tris-HCl (pH 7.2), 0.15 M NaCl, and collected by centrifugation. Cell pellets from each 60 mm dish were resuspended in 1 ml of lysis buffer: 1% NP-40 (polyoxyethylene(9)p-tert-octylphenol), 0.5% sodium deoxycholate, 0.1 M NaCl, 0.01 M Tris-HCl (pH 7.4), 1 mM EDTA and the lysate was cleared by centrifugation for 30 minutes at 100,000 x g.

Immunoprecipitation was carried out by adding 4 ul of heat inactivated human serum, either from a control population or from AIDS patients, to 500 ml of cell lysate. After a one-half hour incubation at 4°C, 80 ul of activated *Staphylococcus aureus* cells (Pansorbin cells, Calbiochem-Behring Corp., La Jolla, CA) was added and incubation was allowed to continue for another one-half hour at 4°C. Immuno-precipitation complexes were collected by centrifugation for 5 minutes at 5000 rpm in a Sorvall A384 rotor at 4°C, and washed once in 1 M NaCl + 0.1% NP-40 + 0.01 M Tris-HCl, pH 7.4 and three times in RIPA buffer (10 mM Tris-HCl, pH 7.2, 0.15 M NaCl, 1% sodium deoxycholate, 1% sodium lauryl sulfate, 1% Triton X-100). Washed immunoprecipitates were resuspended in 80 ul of Laemmli sample buffer, boiled for 1 minute and centrifuged for 5 minutes at 5000 rpm in a Sorvall A384 rotor. Immunoprecipitated proteins present in the supernatant were analyzed by electrophoresis on 10% SDS polyacrylamide gels. After electrophoresis, the gels were stained with Coomassie blue dye, treated with sodium salicylate (30 minutes at room temperature in 1 M sodium salicylate) and dried for fluorography.

It has been suggested that the mature gag proteins (24,000 daltons, p24; 16,000 daltons, p16; 14,000 daltons, p14) are processed from a larger 55,000 dalton precursor protein. FIG. 14 shows that proteins having molecular weights of 67,000 daltons, 55,000 daltons, 40,000 daltons, 34,000 daltons, 32,000 daltons and 24,000 daltons were specifically immunoprecipitated by serum from AIDS patients.

In order to determine whether any precursor/product relationship existed among any of the immunoreactive proteins described in FIG. 18, pulse-chase experiments were performed. Sf9 cells were infected as described above. At 24 hours post infection cells were labeled for 5 minutes with [^{35}S] methionine; the cells were then washed with complete medium and incubated with complete medium for two, four and eight hours. At these times, cells were harvested and immunoprecipitated. Immunoprecipitated proteins were fractionated in 10% SDS polyacrylamide gels as described above. FIG. 19 shows a fluorogram of this experiment. The data suggest that p67, p55, p40, p34 and p32 incorporate label after 5 minutes. After an 8 hour chase, label decreases in p67 and p55 and increases in p34. The amount of radiolabel in p40 remains relatively constant.

7.4.3. IDENTIFICATION OF LAV/HTLV III GAG RELATED PROTEINS BY ELISA

Ten 100 mm dishes, each containing 10^7 Sf9 cells, were infected with Ac-gag1. At 24 hours post infection, cells were harvested, washed twice in 0.01 M Tris-HCl (pH 7.2), 0.15 M NaCl and frozen and thawed three times. Cells were disrupted with 2 ml of PBS containing 0.5% NP-40 on ice for 5 minutes. The lysate was centrifuged at 1000 x g for 10 minutes and the supernatant was removed. 4 ml of carbonate buffer (50 mM sodium carbonate, pH 9.6) was added and the mixture was spun in an Eppendorf centrifuge for 20 minutes. The supernatants were removed and diluted in carbonate buffer

as shown in Table III. These lysates were used to coat 96-well microtiter plates overnight at 4°C (50 ul/well). Three hundred microliters of blocking reagent (5% non-fat dry milk, 0.01% thimerosol, 0.01% antifoam A in 10XPBS) was added to each well and incubated for 45 minutes at room temperature. The blocking reagent was aspirated off, and 50 ul of a 1:100 dilution of human serum in blocking reagent was added to each well. Prior to addition to wells, diluted sera (AIDS patent sera or normal human serum) were incubated at 37°C for 45 minutes.

Each well was allowed to react at 37°C for 1 hour. Wells were then washed three times with sodium chloride + tween-20 (polyoxyethylene sorbitan-monolaurate). One hundred microliters of goat anti-human horseradish peroxidase conjugate (a 1:100 dilution in normal goat serum containing 0.01% thimerosol) was added to each well and allowed to react for 1 hour at 37°C. Plates were washed and 100 ul/well buffered substrate + chromogen reagent were added: buffered substrate is hydrogen peroxide, citric acid, and phosphate buffer; chromogen reagent is tetramethylbenzidine in dimethylsulfoxide. Plates were incubated for 30 minutes at room temperature. The reaction was stopped by adding 100 ul/well 3 N sulfuric acid. The optical absorbance of the reaction mixture was measured at 450 nm with a reference absorbance at 630 nm. The results are presented in Table VIII. TR1, Y-1, CF22C and CF9 are AIDS patients serum; 2, 16, 21 and 48 are normal human serum.

TABLE VIII

ELISA ASSAY ABSORBANCE VALUES

Test Antiserum	Ac-gag 1 LYSATE DILUTION					
	50^{-1}	100^{-1}	200^{-1}	400^{-1}	800^{-1}	1000^{-1}
AIDS:						
TR1	2.619	2.471	2.235	2.076	1.786	1.499
	2.504	2.479	2.216	2.119	1.877	1.500
Y-1	2.502	2.354	2.046	1.564	1.298	1.014
	2.305	2.306	2.006	1.595	1.282	1.078
CF22C	2.486	2.317	1.924	1.393	1.009	0.811
	2.319	2.241	1.838	1.331	1.068	0.848
CF9	2.303	2.110	1.339	0.855	0.636	0.512
	2.209	2.012	1.305	0.850	0.659	0.455
NORMAL:						
2	0.221	0.204	0.312	0.199	0.228	0.222
	0.229	0.187	0.176	0.227	0.208	0.210
16	0.176	0.175	0.217	0.131	0.144	0.129
	0.165	0.135	0.139	0.210	0.175	0.145
21	0.193	0.190	0.216	0.189	0.237	0.165
	0.205	0.176	0.170	0.199	0.197	0.224
48	0.200	0.185	0.217	0.177	0.190	0.134
	0.249	0.169	0.163	0.175	0.173	0.143

Taken together, the results shown in FIG. 17-19 and Table VIII indicate that the recombinant baculoviruses we describe were capable of (a) expressing the inserted LAV/HTLV III gag gene, (b) modifying or processing such gene products, and (c) producing antigenic proteins specifically immunoreactive with AIDS patient serum.

8. EXAMPLE: VACCINIA GAG RECOMBINANTS

In the following example, plasmid vectors were constructed which contained chimeric genes comprising LAV/HTLV III gag coding sequences located downstream with respect to the transcriptional control regions of vaccinia virus. The chimeric gene containing the vaccinia virus 7.5K promoter and the LAV/HTLV III gag sequence was inserted into the genome of vaccinia virus through in vivo recombination. Such recombinant viruses were identified and purified, and viral stocks were prepared from infected tissue culture cells. Immunoreactive LAV/HTLV III gag related proteins were shown to be produced by the recombinant vaccinia viruses in vitro. A detailed description of each step in this embodiment of the invention is presented in the subsections below. The general procedures used for this embodiment are as described in Section 6.1

8.1 CONSTRUCTION OF PLASMID VECTORS CONTAINING A VACCINIA VIRUS PROMOTER LIGATED TO THE CODING SEQUENCES OF LAV/HTLV III GAG GENE

Five plasmid vectors were constructed that contained the vaccinia virus 7.5K promoter ligated to various lengths of the LAV/HTLV III genome containing gag-encoding sequences. The source of LAV/HTLV III gag-encoding sequences was two related subclones of lambda J19 (Wain-Hobson et al., Cell 40: 1985), pKS-5 and pSS-5. Plasmid pKS-5 contains a 3148 base pair SstI to KpnI (nucleotide number 224 to 3,372) fragment of LAV/HTLV III DNA inserted at the corresponding sites of pUC18. Plasmid pSS-5 contains a 5,107 base pair SstI to SalI (nucleotide number 224 to 5221) fragment of LAV/HTLV III DNA inserted at the corresponding sites of pUC18. Various lengths of gag encoding sequences derived from pKS-5 or pSS-5 were inserted into plasmid pGS62, which is identical to PGS20 (Mackett et al., 1984, J. Virol. 49:857-864) with the exception of having a unique EcoRI site located downstream

from the unique SmaI site (see FIG. 20). The LAV/HTLV III specific sequences inserted into pGS62 all start with a ThaI (FnuDII) site (nucleotide 257) located 76 base pairs upstream from the initiation codon of the gag gene. Plasmids pv-gag1, pv-gag2, pv-gag3, pv-gag4 and pv-gag5 contain LAV/HTLV III-specific sequences from this ThaI site through the EcoRI (at 4194), KpnI (at 3372), EcoRV (at 2523), BclI (at 1975), or BglII (at 1642) sites, respectively. The construction of each plasmid, described below, is facilitated by reference to FIG. 20.

Construction of pv-gag1: 5 ug of plasmid pSS-5 DNA was digested to completion with restriction enzymes ThaI and EcoRI. The resulting fragments were resolved on a 1% LMP agarose gel. A 3.9 kbp fragment containing LAV/HTLV III gag coding sequences was isolated and ligated to pGS62 DNA previously digested with SmaI and EcoRI. The ligation mixture was used to transform *E. coli* HB101 and ampicillin-resistant clones were selected. Plasmids from individual colonies were tested for the presence of the 3.9 kbp insert by restriction analysis and the regeneration of the EcoRI site at the ligation junction. The resulting construct contains the entire coding sequence of the gag and the protease (prt) genes, and a major portion of the pol open reading frame/up to the EcoRI site at nucleotide 4194.

Construction of pv-gag2: 5 ug of plasmid pKS-5 was digested to completion with restriction enzymes ThaI and SmaI. The SmaI site of pKS-5 is located adjacent to the KpnI site, which is the junction between the LAV/HTLV III-derived inserted sequence and the pUC18 plasmid multiple cloning sites. The resulting fragments were resolved on a 1% LMP agarose gel, and a 3.2 kbp fragment containing LAV/HTLV III gag sequences was isolated and ligated to pGS62 DNA previously digested with SmaI and treated with CIAP. The ligation mixture was used to transform *E. coli* HB101 and ampicillin-resistant clones were selected. Plasmids from

individual colonies were tested for the presence of the 3.1 kbp fragment, and the orientation of the insertion was determined by restriction analysis. The resulting construct, pv-gag2, contains the entire gag gene, the prt gene and a portion of the pol open reading frame up to the KpnI site at nucleotide 3372.

Construction of pv-gag3 was similar to that of pv-gag2, with the exception that a 2.3 kbp fragment generated by ThaI and EcoRV digests of pKS-5 was used for the insertion into pGS62 at the SmaI site. The final construct, pv-gag3, contains the entire gag and prt genes and a small part of pol fused to the 3' portion of the vaccinia virus TK gene.

Construction of pv-gag4: 5 ug of plasmid pSS-5 DNA was digested to completion with SstI and BclI. The resulting fragment containing the LAV/HTLV III gag sequence was purified. This fragment was ligated to plasmid vector pIC19R (Marsh et al., 1984, Gene 32: 481-485) previously cut with SstI and BamHI to construct an intermediate plasmid. Insertion of the gag sequence into the BamHI site of pIC19R results in the juxtaposition of the BclI site in the LAV/HTLV III sequence to the EcoRI site in this multiple-site cloning vector. Digestion of this intermediate construct with restriction enzymes ThaI and EcoRI generated a 1.72 kbp fragment that could be readily inserted into plasmid pGS62 at the SmaI and EcoRI sites. The final construct, pv-gag4, contains the entire LAV/HTLV III gag gene and a portion of the prt gene fused to the 3' portion of the vaccinia virus TK gene.

Construction of plasmid pv-gag5 made use of the same two-step strategy as pv-gag4. Five micrograms of pSS-5 were digested with restriction enzymes SstI and BglII. A 1.42 kbp fragment containing LAV/HTLV III gag sequence was isolated and purified on a 1% LMP agarose gel and inserted into plasmid pIC19R at the SstI and BglII sites. Ligation of the gag sequence at the BglII site to its corresponding site on

pIC19R allows (a) the generation of a stop codon in phase with the gag open reading frame, and (b) the juxtaposition of the BglII site to the adjacent EcoRI site on the multiple cloning-site plasmid. This intermediate construct was then digested with ThaI and EcoRI. A 1.39 kbp fragment containing LAV/HTLV III gag sequences was purified and ligated to plasmid pGS62 at SmaI and EcoRI sites. The resulting plasmid, pv-gag5, contains most but not all of the coding sequence of gag (up to the BglII site) ligated to an in-frame translational stop sequence.

8.2 CONSTRUCTION OF RECOMBINANT VACCINIA VIRUSES CONTAINING CHIMERIC LAV/HTLV III GAG GENES

Insertion of the chimeric LAV/HTLV III gag genes from plasmid vectors pv-gag1, pv-gag2, pv-gag3, pv-gag4 and pv-gag5 into the vaccinia virus genome was achieved by the same protocol used for the construction of v-env5 and v-env2 (see section 6.3). In the examples that follow, all recombinant viruses were constructed with the plaque-purified New York City Board of Health strain (v-NY, see section 6.1.1). TK^- recombinants were selected and plaque-purified three times before being expanded into viral stocks that were used for subsequent characterizations. Recombinant viruses derived from pv-gag1, pv-gag2, pv-gag3, pv-gag4 and pv-gag5 were designated as v-gag1NY, v-gag2NY, v-gag3NY, v-gag4NY and v-gag5NY, respectively.

8.3 EXPRESSION OF LAV/HTLV III GAG RELATED PROTEINS IN TISSUE CULTURE CELLS INFECTED BY RECOMBINANT VACCINIA VIRUSES

The recombinant vaccinia viruses carrying the chimeric LAV/HTLV III gag gene described above were capable of expressing LAV/HTLV III gag related proteins upon infection of cells in tissue culture. Some of these recombinants were able to process the precursor gag protein, p55, into mature proteins p25 and p18. These proteins were found to be

immunoreactive with AIDS patient serum and/or monoclonal antibodies against gag proteins p25 or p18.

Confluent BSC-40 cells in a 60 mm dish were infected at a moi of 10 by parental_type vaccinia virus (v-NY) or with its recombinant derivatives, v-gag1NY, v-gag2NY, v-gag3NY, v-gag4NY or v-gag5NY. Infection was allowed to proceed for 12 hours, at which time the cells were harvested, washed once with PBS and collected by centrifugation. Infected cell pellets were resuspended in 0.3 ml of Laemmli sample buffer and lysed by boiling for 4 minutes. Total cellular protein in a 20 ul aliquot of cell lysate was resolved by electrophoresis on a 15% SDS polyacrylamide gel. A sample of purified LAV/HTLV III virion and an aliquot of mock infected cell lysate were included as controls.

The contents of the gel were electro-transferred to a sheet of nitrocellulose filter. The filter was first reacted either with AIDS patient serum, or with a combination of monoclonal antibodies against gag proteins p25 and p18. After extensive washings, the filter was then reacted either with alkaline phosphatase-conjugated (AP-conjugated) goat anti-human immunoglobulin antibodies in cases where patient serum was used, or with AP-conjugated goat anti-mouse immunoglobulin antibodies in cases where mouse monoclonal antibodies were used. Conditions for the first and second antibody reactions as well as for the washings were as described in section 6.4.1. The final washing after the second antibody reaction was done in 0.1 M Tris (pH 9.5) with 0.1 M NaCl and 5 mM $MgCl_2$. Antibody bound to antigens on the filter was detected by reacting the filter with a solution containing 0.1 M Tris (pH 9.5), 0.1 M NaCl, 5 mM $MgCl_2$, 0.33 mg/ml bromo-chloro-indolyl phosphate, and 0.17 mg/ml of nitro-blue-tetrazolium. After filters were reacted with the chromogens, they were rinsed in water, air-dried and photographed.

The results of this analysis are shown in Fig. 21. Recombinants v-gag1NY and v-gag2NY contain the entire gag and prt genes. These recombinants expressed a family of LAV/HTLV III related proteins that co-migrated with the major gag proteins of LAV/HTLV III, p55, p40, p25 and p18. These proteins were immunoreactive with both AIDS patient serum (Fig. 21B) and mouse monoclonal antibodies against p25 and p18 (Fig. 21A). Recombinant v-gag3NY contains both gag and prt genes, but its pol open reading frame is ligated in phase with the carboxy terminal portion of the vaccinia TK gene. V-gag3NY was also capable of expressing the primary gag gene product, p55. However, the processing of p55 appeared to be less efficient than v-gag1NY and v-gag2NY, since there was much less p25 and p18 relative to p55 in cell lysates infected with v-gag3NY. Recombinant v-gag4NY contains the entire gag gene, but only part of the prt gene. It synthesized a LAV/HTLV III related protein similar in size to p55. Recombinant v-gag5NY contains only part of the gag gene and expressed a truncated protein of 43 kD (FIG. 21C). Although both v-gag4NY and v-gag5NY did not appear to process their primary gag related protein efficiently, their products were immunoreactive to monoclonal antibodies against p25 and p18. In summary, despite the differences in their abilities to process, all five recombinants were able to express immunoreactive proteins that contained major epitopes of the LAV/HTLV III core proteins.

9. EXAMPLE: BACULOVIRUS ENVELOPE RECOMBINANTS

In the following example, a plasmid vector was constructed containing a chimeric gene comprising LAV/HTLV III env coding sequences located downstream with respect to the transcriptional control sequences of AcNPV. The chimeric gene containing the AcNPV polyhedrin promoter and the LAV/HTLV III env coding sequence was inserted into the genome of AcNPV through *in vivo* recombination. Recombinant virus

were identified and purified and viral stocks were prepared from infected cell supernatants. Immunoreactive LAV/HTLV III env related protein was shown to be produced by the recombinant baculovirus in vitro. A detailed description of each step in this embodiment of the invention is presented in the subsections below. The general procedures used for this embodiment are as described in Section 7.1.

9.1 CONSTRUCTION OF PLASMID VECTORS CONTAINING AcNPV PROMOTER LIGATED TO THE CODING SEQUENCES OF LAV/HTLV III ENV GENE

LAV/HTLV III env coding sequence was purified from pv-env5 (see section 6.3), which was used for the construction of recombinant vaccinia virus v-env5 that was shown to express env related proteins. The env coding region in pv-env5, together with 96 base pairs 5' proximal and 223 base pairs 3' proximal untranslated sequences, was flanked by BamHI sites on both ends. Partial digestion of this plasmid (which also contains an internal BamHI site) with restriction enzyme BamHI generated a 2.9 kbp fragment containing only LAV/HTLV III specific sequences. This fragment (about 0.5 ug) was resolved by and purified from a 1% LMP agarose gel, and was ligated to 0.5 ug of plasmid vector pAc610, which was previously linearized with BamHI and treated with CIAP. The ligation mixture was used to transform E. coli strain MC1000 and ampicillin-resistant colonies were selected. Plasmid DNA from individual transformants was tested for the presence of LAV/HTLV III env sequences (i.e. generation of the 2.3 Kbp and 0.54 Kbp fragments by BamHI digestion) and for the orientation of the insert. Plasmid with LAV/HTLV III env sequence inserted in the correct orientation was purified and designated pAc-env5. Its structure is depicted in Fig. 22.

9.2 CONSTRUCTION OF RECOMBINANT BACULOVIRUS CONTAINING CHIMERIC LAV/HTLV III ENV GENE

Insertion of the chimeric LAV/HTLV III env gene into the AcNPV genome is achieved by *in vivo* recombination, as explained in section 7.3. Transfection of AcNPV DNA (1 mg), pAc-env5 DNA (5 mg) and calf-thymus DNA (15 mg) into Sf9 cells was done as described in the same section. After five days of incubation at 28°C with 4 ml of complete medium, supernatants were collected and clarified at 1000 x g for 10 minutes. The virus stock was titered on Sf9 cells, and recombinant virus was first identified by the plaque hybridization assay of Summers, M.D. and Smith, G.E. as follows:

Sf9 cells were seeded onto 60 x 15 mm culture plates at a density of 2.5 x 10^6 cells per plate in serum-free TNM-FH medium. After cells attached, media was removed and 1 ml of diluted virus inoculum was added to each plate. At the end of the 1 hour incubation at 27°C, all inoculum was removed from each plate and 4 ml of LMP agar overlay was slowly added to the edge of each plate. After overlay solidified, plates were incubated in a humid environment for 4-6 days, or until plaques were well-formed. Plates were then allowed to dry overnight or longer by leaving them in an unhumidified environment. When sufficiently dry, the agarose/overlays were removed and a dry nitrocellulose filter was placed on top of the cells remaining in the plate. A a 47 mm circle of Whatman 3 MM paper was saturated with solution A (0.05 M Tris-HCl, pH 7.4, 0.15 M NaCl) and placed on top of the nitrocellulose filter in the plate. After blotting, the nitrocellulose filter was carefully removed and placed (cell side up) on a Whatman filter saturated with Solution B (0.5 N NaOH, 1.5 M NaCl). After 2-3 minutes, the nitrocellulose filter was air-dried on paper towels and transferred to a Whatman filter saturated with solution C (1.0 M Tris-HCl, pH 7.4, 1.5 M NaCl). After 2-3 minutes, the nitrocellulose

filter was air-dried on paper towels, and washed by gentle immersion into a petri dish filled with solution D (0.3 M NaCl, 0.03 M sodium citrate). It was then removed and dried on paper towels, and baked at 80°C for 2 hours under vacuum. The filter was hybridized to ^{32}P-labeled pRS-3, as probe. Recombinant viruses were picked and titered again on Sf9 cells. Non-occluded plaques were identified by visual inspection. These plaque-purified three more times and were used to prepare viral stocks for subsequent studies.

9.3 EXPRESSION OF LAV/HTLV III ENV RELATED PROTEINS IN TISSUE CULTURE CELLS INFECTED BY RECOMBINANT BACULOVIRUS Ac-env5

The recombinant AcNPV carrying the chimeric LAV/HTLV III env gene was shown to be capable of expressing LAV/HTLV III env related proteins upon infection of cells in tissue culture. These proteins were found to be immunoreactive with AIDS patient serum as well as with monoclonal antibodies that define LAV/HTLV III envelope glycoproteins gp110 and gp41.

Sf9 cells (1×10^7) seeded onto a 100 mm dish were infected with wild-type AcNPV, or its recombinant Ac-env5, at a moi of 5. At 28 hours post infection, the media was replaced by methionine-free medium with no serum supplements, and incubated for 30 minutes. After this period, the medium was replaced by 2 ml of methionine-free medium containing 100 uCi/ml of [^{35}S] methionine, and labeling was allowed to proceed for 2 hours at 28°C. At the end of the labeling period, cells were washed twice with PBS and resuspended in 1 ml of lysis buffer as described in Section 6.4.2. Immunoprecipitation was carried out as described in Section 6.4.2., using AIDS patient serum as well as mouse monoclonal antibodies against gp110 or gp41.

The results of this analysis, as shown in Fig. 23, indicate that the recombinant virus Ac-env5 synthesized predominantly one LAV/HTLV III envelope related protein of

150 kD. Because it comigrates with gp150 synthesized by vaccinia recombinant v-env5 and was immunoreactive with AIDS patient serum as well as monoclonal antibodies to gp110 and gp41, this protein represents the glycosylated precursor envelope protein gp150. The processing of this molecule was not efficient, since no gp110 or gp41 was detected in the two hour labeling period. However, since the major epitopes of both gp110 and gp41 are present on the precursor, this protein is potentially valuable both as a diagnostic reagent and as an immunogen.

10. DEPOSIT OF MICROORGANISMS

The following E. coli strains carrying the listed plasmids have been deposited with the Agricultural Research Culture Collection (NRRL), Peoria, IL and have been assigned the following accession numbers:

E. Coli	Strain	Plasmid	Accession Number
K12,	MC1000	pv-env1	NRRL B-18003
K12,	MC1000	pv-env2	NRRL B-18004
K12,	MC1000	pv-env5	NRRL B-18005
K12,	MC1000	pv-env7	NRRL
K12,	HB101	pv-gag1	NRRL
K12,	HB101	pAc-gag1	NRRL B-18105
K12,	NF1829	pAc-env5	NRRL

The following recombinant viruses have been deposited with the American Type Culture Collection, Rockville, MD, and have been assigned the listed accession numbers:

Recombinant Virus	Accession Number
v-env2	ATCC VR 2114
v-env5	ATCC VR 2113
v-env7	ATCC
v-env5NY	ATCC
v-gag1NY	ATCC
Ac-gag1	ATCC VR 2147
Ac-env5	ATCC

The present invention is not to be limited in scope by the microorganisms and viruses deposited since the deposited embodiment is intended as a single illustration of one aspect of the invention and any microorganisms or viruses which are functionally equivalent are within the scope of this invention. Indeed, various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and accompanying drawings. Such modifications are intended to fall within the scope of the appended claims.

It is also to be understood that all base pair sizes given for nucleotides are approximate and are used for purposes of description.

WHAT IS CLAIMED IS:

1. A recombinant virus the genome of which comprises a nucleotide sequence encoding an epitope of LAV/HTLV III, or an antigenic portion thereof which is under the control of a second nucleotide sequence that regulates gene expression so that a peptide or protein related to the epitope of LAV/HTLV III is expressed in a host infected with the virus.

2. The recombinant virus according to claim 1 in which the nucleotide sequence encoding the epitope of LAV/HTLV III comprises that of the envelope gene of LAV/HTLV III or any portion thereof encoding an antigenic peptide or protein.

3. The recombinant virus according to claim 2 in which the envelope gene nucleotide sequence comprises the nucleotide sequence substantially as depicted in FIG. 2 from nucleotide number 5767 to 8549, or any portion thereof encoding an antigenic peptide or protein.

4. The recombinant virus according to claim 1 in which the peptide or protein expressed in the infected host is related to an envelope epitope of LAV/HTLV III, or any antigenic portion thereof.

5. The recombinant virus according to claim 4 in which the envelope epitope comprises a peptide or protein having an amino acid sequence substantially as depicted in FIG. 2 from amino acid residue number 1 to 861, or any antigenic portion thereof.

6. The recombinant virus according to claim 1, 2, 3, 4 or 5 comprising an enveloped virus.

7. The recombinant virus according to claim 6 comprising a vaccinia virus.

8. Recombinant vaccinia virus v-env5 as deposited with the ATCC and assigned accession number VR 2113 or a mutant, recombinant or genetically engineered derivative thereof.

9. Recombinant vaccinia virus v-env2 as deposited with the ATCC and assigned accession number VR 2114 or a mutant, recombinant or genetically engineered derivative thereof.

10. Recombinant vaccinia virus v-env7 as deposited with the ATCC and assigned accession number VR or a mutant, recombinant or genetically engineered derivative thereof.

11. Recombinant vaccinia virus v-env5NY as deposited with the ATCC and assigned accession number VR or a mutant, recombinant or genetically engineered derivative thereof.

12. The recombinant virus according to claim 1, 2, 3, 4 or 5 comprising a baculovirus.

13. The recombinant virus according to claim 12 comprising a nuclear polyhedrosis virus.

14. Recombinant baculovirus Ac-env5 as deposited with the ATCC and assigned accession number VR or a mutant, recombinant or genetically engineered derivative thereof.

15. The recombinant virus according to claim 1, 2, 3, 4 or 5 comprising a naked virus.

16. The recombinant virus according to claim 15 comprising an adenovirus.

17. The recombinant virus according to claim 1, 2, 3, 4 or 5 comprising a bacteriophage.

18. The recombinant virus according to claim 1 in which the nucleotide sequence encoding the epitope of LAV/HTLV III comprises that of the gag gene of LAV/HTLV III or any portion thereof encoding an antigenic peptide or protein.

19. The recombinant virus according to claim 18 in which the gag gene nucleotide sequence comprises the nucleotide sequence substantially as depicted in FIG. 14 from nucleotide number 340 to 1835, or any portion thereof encoding an antigenic peptide or protein.

20. The recombinant virus according to claim 1 in which the peptide or protein expressed in the infected host is related to an epitope of a core protein of LAV/HTLV III, or any antigenic portion thereof.

21. The recombinant virus according to claim 20 in which the epitope comprises a peptide or protein having an amino acid sequence substantially as depicted in FIG. 14 from amino acid residue number 1 to 500, or any antigenic portion thereof.

22. The recombinant virus according to claim 18, 19, 20 or 21 comprising an enveloped virus.

23. The recombinant virus according to claim 22 comprising a vaccinia virus.

24. Recombinant vaccinia virus v-gag1NY as deposited with the ATCC and assigned accession number VR , or a mutant, recombinant or genetically engineered derivative thereof.

25. The recombinant virus according to claim 18, 19, 20 or 21 comprising a baculovirus.

26. The baculovirus according to claim 25 comprising a nuclear polyhedrosis virus.

27. Recombinant baculovirus Ac-gag1 as deposited with the ATCC and assigned accession number VR 2147 or a mutant, recombinant, or genetically engineered derivative thereof.

28. The recombinant virus according to claim 18, 19, 20 or 21 comprising a naked virus.

29. The recombinant virus according to claim 28 comprising an adenovirus.

30. The recombinant virus according to claim 18, 19, 20 or 21 comprising a bacteriophage.

31. A substantially pure antigenic peptide or protein related to an epitope of LAV/HTLV III.

32. The peptide or protein according to claim 31 in which the epitope comprises an envelope glycoprotein epitope of LAV/HTLV III.

33. The peptide or protein according to claim 32 having an amino acid sequence comprising the amino acid sequence substantially as depicted in FIG. 2 from amino acid residue number 1 to 861 or any antigenic portion thereof.

34. The peptide or protein of claim 32 or 33 in which the peptide or protein was purified from a cultured cell containing a nucleotide sequence encoding the peptide or protein which is under the control of a second nucleotide

sequence that regulates gene expression so that the peptide or protein is expressed by the cultured cell.

35. The peptide or protein of claim 34 in which the cultured cell comprises a microorganism.

36. The peptide or protein of claim 35 in which the microorganism comprises a bacterium.

37. The peptide or protein of claim 35 in which the microorganism comprises a yeast.

38. The peptide or protein of claim 34 in which the cultured cell comprises an animal cell line.

39. The peptide or protein of claim 38 produced by animal cells infected with recombinant vaccinia virus v-env5 as deposited with the ATCC and assigned accession number VR 2113 or a mutant, recombinant or genetically engineered derivative thereof.

40. The peptide or protein of claim 38 produced by animal cells infected with recombinant vaccinia virus v-env2 as deposited with the ATCC and assigned accession number VR 2114 or a mutant, recombinant or genetically engineered derivative thereof.

41. The peptide or protein of claim 38 produced by animal cells infected with recombinant vaccinia virus v-env7 as deposited with the ATCC and assigned accession number VR or a mutant, recombinant or genetically engineered derivative thereof.

42. The peptide or protein of claim 38 produced by animal cells infected with recombinant vaccinia virus v-

env5NY as deposited with the ATCC and assigned accession number VR or a mutant, recombinant or genetically engineered derivative thereof.

43. The peptide or protein of claim 34 in which the cultured cell comprises an insect cell line.

44. The peptide or protein of claim 43 produced by insect cells infected with recombinant baculovirus Ac-env5 as deposited with the ATCC and assigned accession number VR or a mutant, recombinant, or genetically engineered derivative thereof.

45. The peptide or protein according to claim 31 in which the epitope comprises a core protein epitope of LAV/HTLV III.

46. The peptide or protein according to claim 45 having an amino acid sequence comprising the amino acid sequence substantially as depicted in FIG. 14 from amino acid residue number 1 to 500 or any antigenic portion thereof.

47. The peptide or protein of claim 45 or 46 in which the peptide or protein was purified from a cultured cell containing a nucleotide sequence encoding the peptide or protein which is under the control of a second nucleotide sequence that regulates gene expression so that the peptide or protein is expressed by the cultured cell.

48. The peptide or protein of claim 47 in which the cultured cell comprises a microorganism.

49. The peptide or protein of claim 48 in which the microorganism comprises a bacterium.

50. The peptide or protein of claim 48 in which the microorganism comprises a yeast.

51. The peptide or protein of claim 47 in which the cultured cell comprises an animal cell line.

52. The peptide or protein of claim 51 produced by animal cells infected with recombinant vaccinia virus v-gag1NY as deposited with the ATCC and assigned accession number VR or a mutant, recombinant or genetically engineered derivative thereof.

53. The peptide or protein of claim 47 in which the cultured cell comprises an insect cell line.

54. The peptide or protein of claim 53 produced by insect cells infected with recombinant baculovirus Ac-gag1 as deposited with the ATCC and assigned accession number VR 2147 or a mutant, recombinant, or genetically engineered derivative thereof.

55. The peptide or protein of claim 31, 32, 33, 45, or 46 in which the peptide or protein was chemically synthesized.

56. A live virus vaccine formulation comprising a virus of claim 1, 2, 3, 4 or 5 in which the virus is infectious without causing disease in a host to be vaccinated.

57. The live virus vaccine formulation according to claim 56 in which the virus comprises an enveloped virus.

58. The live virus vaccine formulation according to claim 57 in which the enveloped virus comprises a vaccinia virus.

59. A live virus vaccine formulation comprising an infectious dose of the vaccinia virus v-env5 of claim 8.

60. A live virus vaccine formulation comprising an infectious dose of the vaccinia virus v-env2 of claim 9.

61. A live virus vaccine formulation comprising an infectious dose of the vaccinia virus v-env7 of claim 10.

62. A live virus vaccine formulation comprising an infectious dose of the vaccinia virus v-env5NY of claim 11.

63. The live virus vaccine formulation according to claim 56 in which the virus comprises a naked virus.

64. The live virus vaccine formulation according to claim 63 in which the naked virus comprises an adenovirus.

65. A multivalent live virus vaccine formulation comprising a recombinant virus of claim 2, 3, 4 or 5 and a recombinant virus of claim 18, 19, 20 or 21, in which both recombinant viruses are infectious without causing disease in the host to be vaccinated.

66. The multivalent vaccine of claim 65 in which the each recombinant virus comprises an enveloped virus.

67. The multivalent live virus vaccine of claim 66 in which each enveloped virus comprises a vaccinia virus.

68. A multivalent vaccine formulation comprising an infectious dose of:

(a) the vaccinia virus v-env5 of claim 8, v-env2 of claim 9, v-env7 of claim 10 or v-env5NY of claim 11; and

(b) the vaccinia virus v-gag1NY of claim 24.

69. An inactivated virus vaccine formulation comprising an effective dose of the virus of claim 1, 2, 3, 4 or 5, in a non-infectious state mixed with a pharmaceutical carrier.

70. An inactivated virus vaccine formulation comprising an effective dose of the enveloped virus of claim 6 in a non-infectious state mixed with a pharmaceutical carrier.

71. An inactivated virus vaccine formulation comprising an effective dose of the vaccinia virus of claim 7 in a non infectious state mixed with a phamaceutical carrier.

72. An inactivated virus vaccine formulation comprising an effective dose of the vaccinia virus v-env5 of claim 8 in a non-infectious state mixed with a pharmaceutical carrier.

73. An inactivated virus vaccine formulation comprising an effective dose of the vaccinia virus v-env2 of claim 9 in a non-infectious state mixed with a pharmaceutical carrier.

74. An inactivated virus vaccine formulation comprising an effective dose of the vaccinia virus v-env7 of claim 10 in a non-infectious state mixed with a pharmaceutical carrier.

75. An inactivated virus vaccine formulation comprising an effective dose of the vaccinia virus v-env5NY of claim 11 in a non-infectious state mixed with a pharmaceutical carrier.

76. A multivalent inactivated virus vaccine formulation comprising an effective dose of:

(a) the recombinant virus of claim 2, 3, 4 or 5 which expresses the envelope related epitope of LAV/HTLV III; and

(b) the recombinant virus of claim 18, 19, 20 or 21 which expresses the core related epitope of LAV/HTLV III,

in which each recombinant virus is in a non-infectious state mixed with a pharmaceutical carrier.

77. The multivalent inactivated virus vaccine formulation of claim 76 in which each recombinant virus comprises an enveloped virus.

78. The multivalent inactivated virus vaccine formulation of claim 77 in which each enveloped virus comprises a vaccinia virus.

79. A multivalent inactivated virus vaccine formulation comprising an effective dose of:

(a) the vaccinia virus v-env5 of claim 8, v-env2 of claim 9, v-env7 of claim 10, or v-env5NY of claim 11; and

(b) the vaccinia virus v-gag1NY of claim 24.

80. A subunit vaccine formulation in which the immunogen comprises an effective dose of the peptide or protein of claim 32 or 33 mixed with a pharmaceutical carrier.

81. A subunit vaccine formulation in which the immunogen comprises an effective dose of the peptide or protein of claim 34 mixed with a pharmaceutical carrier.

82. A subunit vaccine formulation in which the immunogen comprises an effective dose of the peptide or protein of claim 35 mixed with a pharmaceutical carrier.

83. A subunit vaccine formulation in which the immunogen comprises an effective dose of the peptide or protein of claim 36 mixed with a pharmaceutical carrier.

84. A subunit vaccine formulation in which the immunogen comprises an effective dose of the peptide or protein of claim 37 mixed with a pharmaceutical carrier.

85. A subunit vaccine formulation in which the immunogen comprises an effective dose of the peptide or protein of claim 38 mixed with a pharmaceutical carrier.

86. A subunit vaccine formulation in which the immunogen comprises an effective dose of the peptide or protein of claim 39 mixed with a pharmaceutical carrier.

87. A subunit vaccine formulation in which the immunogen comprises an effective dose of the peptide or protein of claim 40 mixed with a pharmaceutical carrier.

88. A subunit vaccine formulation in which the immunogen comprises an effective dose of the peptide or protein of claim 41 mixed with a pharmaceutical carrier.

89. A subunit vaccine formulation in which the immunogen comprises an effective dose of the peptide or protein of claim 42 mixed with a pharmaceutical carrier.

90. A subunit vaccine formulation in which the immunogen comprises an effective dose of the peptide or protein of claim 43 mixed with a pharmaceutical carrier.

91. A subunit vaccine formulation in which the immunogen comprises an effective dose of the peptide or protein of claim 44 mixed with a pharmaceutical carrier.

92. A multivalent subunit vaccine formulation comprising an effective dose of:

(a) a first peptide or protein of claim 32 or 33, and

(b) a second peptide or protein of claim 45 or 46,

mixed with a pharmaceutical carrier.

93. A multivalent subunit vaccine formulation comprising an effective dose of:

(a) a first peptide of claim 32 or 33, and

(b) a second peptide or protein of claim 47,

mixed with a pharmaceutical carrier.

94. A multivalent subunit vaccine formulation comprising an effective dose of:

(a) a first peptide of claim 32 or 33, and

(b) a second peptide or protein of claim 48,

mixed with a pharmaceutical carrier.

95. A multivalent subunit vaccine formulation comprising an effective dose of:

(a) a first peptide of claim 32 or 33, and

(b) a second peptide or protein of claim 49,

mixed with a pharmaceutical carrier.

96. A multivalent subunit vaccine formulation comprising an effective dose of:

(a) a first peptide of claim 32 or 33, and

(b) a second peptide or protein of claim 50,

mixed with a pharmaceutical carrier.

97. A multivalent subunit vaccine formulation comprising an effective dose of:

(a) a first peptide of claim 32 or 33, and

(b) a second peptide or protein of claim 51,

mixed with a pharmaceutical carrier.

98. A multivalent subunit vaccine formulation comprising an effective dose of:

(a) a first peptide of claim 32 or 33, and

(b) second peptide or protein of claim 52,

mixed with a pharmaceutical carrier.

99. A multivalent subunit vaccine formulation comprising an effective dose of:

(a) a first peptide of claim 32 or 33, and

(b) second peptide or protein of claim 53,

mixed with a pharmaceutical carrier.

100. A multivalent subunit vaccine formulation comprising an effective dose of:

(a) a first peptide of claim 32 or 33, and

(b) second peptide or protein of claim 54,

mixed with a pharmaceutical carrier.

101. A recombinant DNA vector comprising pv-envl.

102. A unicellular organism containing the recombinant DNA vector of claim 101.

103. A bacterium containing the recombinant DNA vector of claim 101.

104. The bacterium of claim 103 comprising Escherichia coli as deposited with the NRRL and assigned accession number B-18003 or a mutant, recombinant or genetically engineered derivative thereof.

105. A recombinant DNA vector comprising pv-env2.

106. A unicellular organism containing the recombinant DNA vector of claim 105.

107. A bacterium containing the recombinant DNA vector of claim 105.

108. The bacterium of claim 107 comprising Escherichia coli as deposited with the NRRL and assigned accession number B-18004, or a mutant, recombinant or genetically engineered derivative thereof.

109. A recombinant DNA vector comprising pv-env5.

110. A unicellular organism containing the recombinant DNA vector of claim 109.

111. A bacterium containing the recombinant DNA vector of claim 109.

112. The bacterium of claim 111 comprising Escherichia coli as deposited with the NRRL and assigned accession number B-18005, or a mutant, recombinant or genetically engineered derivative thereof.

113. A recombinant DNA vector comprising pv-env7.

114. A unicellular organism containing the recombinant DNA vector of claim 113.

115. A bacterium containing the recombinant DNA vector of claim 113.

116. The bacterium of claim 115 comprising Escherichia coli as deposited with the NRRL and assigned accession number B- , or a mutant, recombinant or genetically engineered derivative thereof.

117. A recombinant DNA vector comprising pv-gag1.

118. A unicellular organism containing the recombinant DNA vector of claim 117.

119. A bacterium containing the recombinant DNA vector of claim 117.

120. The bacterium of claim 119 comprising Escherichia coli as deposited with the NRRL and assigned accession number B- , or a mutant, recombinant or genetically engineered derivative thereof.

121. A recombinant DNA vector comprising pAc-gag1.

122. A unicellular organism containing the recombinant DNA vector of claim 121.

123. A bacterium containing the recombinant DNA vector of claim 121.

124. The bacterium of claim 123 comprising Escherichia coli as deposited with the NRRL and assigned accession number B-18105, or a mutant, recombinant or genetically engineered derivative thereof.

125. A recombinant DNA vector comprising pAc-env5.

126. A unicellular organism containing the recombinant DNA vector of claim 125.

127. A bacterium containing the recombinant DNA vector of claim 125.

128. The bacterium of claim 127 comprising Escherichia coli as deposited with the NRRL and assigned accession number B- , or a mutant, recombinant or genetically engineered derivative thereof.

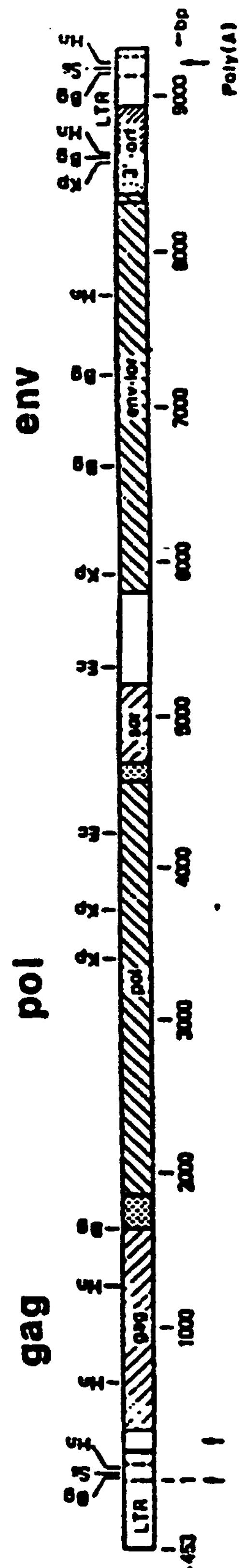
gag
pol
env
LTR
gag
pol
sor
env-lor
3'-orf
LTR
-453
1000
2000
3000
4000
5000
6000
7000
8000
9000
~bp
Poly(A)
Bg
Hi
Kp
Ec

FIG. 1

FIG. 2

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LYMPHADENOPATHY-ASSOCIATED VIRUS (LAV)

    CATAATAAGAATTCTGCAACAACTGCTGTTTATCCATTTCAGAATTGGGTGTCGACATAG   5340
            ECO RI

    CAGAATAGGCGTTACTCAACAGAGGAGAGCAAGAAATGGAGCCAGTAGATCCTAGACTAG   5400

    AGCCCTGGAAGCATCCAGGAAGTCAGCCTAAAACTGCTTGTACCACTTGCTATTGTAAAA   5460

    AGTGTTGCTTTCATTGCCAAGTTTGTTTCACAACAAAAGCCTTAGGCATCTCCTATGGCA   5520

    GGAAGAAGCGGAGACAGCGACGAAGACCTCCTCAAGGCAGTCAGACTCATCAAGTTTCTC   5580

    TATCAAAGCAGTAAGTAGTACATGTAATGCAACCTATACAAATAGCAATAGCAGCATTAG   5640

    TAGTAGCAATAATAATAGCAATAGTTGTGTGGTCCATAGTAATCATAGAATATAGGAAAA   5700
                                   AvaII

    TATTAAGACAAAGAAAAATAGACAGGTTAATTGATAGACTAATAGAAAGAGCAGAAGACA   5760

    GTGGCAATGAGAGTGAAGGAGAAATATCAGCACTTGTGGAGATGGGGGTGGAAATGGGGC   5820
  1         MetArgValLysGlulysTyrGlnHisLeuTrpArgTrpGlyTrpLysTrpGly

    ACCATGCTCCTTGGGATATTGATGATCTGTAGTGCTACAGAAAAATTGTGGGTCACAGTC   5880
 19 ThrMetLeuLeuGlyIleLeumetIleCysSerAlaThrGluLysleuTrpValThrVal

             KpnI
 39 TATTATGGGGTACCTGTGTGGAAGGAAGCAACCACCACTCTATTTTGTGCATCAGATGCT   5940
    TyrTyrGlyValProValTrpLysGluAlaThrThrThrleuPheCysAlaSerAspAla

    AAAGCATATGATACAGAGGTACATAATGTTTGGGCCACACATGCCTGTGTACCCACAGAC   6000
 59 LysAlaTyrAspThrGluValHisAsnValTrpAlaThrHisAlaCysValProThrAsp

    CCCAACCCACAAGAAGTAGTATTGGTAAATGTGACAGAAAATTTTAACATGTGGAAAAAT   6060
 79 ProAsnProGlnGluValValLeuValAsnValThrGluAsnPheAsnMetTrpLysAsn

    GACATGGTAGAACAGATGCATGAGGATATAATCAGTTTATGGGATCAAAGCCTAAAGCCA   6120
 99 AspMetValGluGlnMetHisGluAspIleIleSerLeuTrpAspGlnSerLeuLysPro

    TGTGTAAAATTAACCCCACTCTGTGTTAGTTTAAAGTGCACTGATTTGGGGAATGCTACT   6180
119 CysVallysLeuThrProLeuCysValSerLeuLysCysThrAspLeuGlyAsnAlaThr

    AATACCAATAGTAGTAATACCAATAGTAGTAGCGGGGAAATGATGATGGAGAAAGGAGAG   6240
139 AsnThrAsnSerSerAsnThrAsnSerSerSerGlyGluMetMetMetGluLysGlyGlu

    ATAAAAAACTGCTCTTTCAATATCAGCACAAGCATAAGAGGTAAGGTGCAGAAAGAATAT   6300
159 IleLysAsnCysSerPheAsnIleSerThrSerIleArgGlyLysValGlnLysGluTyr

    GCATTTTTTTATAAACTTGATATAATACCAATAGATAATGATACTACCAGCTATACGTTG   6360
179 AlaPhePheTyrLysLeuAspIleIleProIleAspAsnAspThrThrSerTyrThrLeu
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FIG.2 (con't)

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                                 STU I
    ACAAGTTGTAACACCTCAGTCATTACACAGGCCTGTCCAAAGGTATCCTTTGAGCCAATT  6420
199 ThrSerCysAsnThrSerValIleThrGlnAlaCysProLysValSerPheGluProIle

    CCCATACATTATTGTGCCCCGGCTGGTTTTGCGATTCTAAAATGTAATAATAAGACGTTC  6480
219 ProIleHisTyrCysAlaProAlaGlyPheAlaIleLeuLysCysAsnAsnLysThrPhe

           AvaII
    AATGGAACAGGACCATGTACAAATGTCAGCACAGTACAATGTACACATGGAATTAGGCCA  6540
239 AsnGlyThrGlyProCysThrAsnValSerThrValGlnCysThrHisGlyIleArgPro

    GTAGTATCAACTCAACTGCTGTTGAATGGCAGTCTAGCAGAAGAAGAGGTAGTAATTAGA  6600
259 ValValSerThrGlnLeuLeuLeuAsnGlySerLeuAlaGluGluGluValValIleArg

    TCTGCCAATTTCACAGACAATGCTAAAACCATAATAGTACAGCTGAACCAATCTGTAGAA  6660
279 SerAlsAsnPheThrAspAsnAlaLysThrIleIleValGlnLeuAsnGlnSerValGlu

    ATTAATTGTACAAGACCCAACAACAATACAAGAAAAAGTATCCGTATCCAGAGGGGACCA  6720
299 IleAsnCysThrArgProAsnAsnAsnThrArgLysSerIleArgIleGlnArgGlyPro

    GGGAGAGCATTTGTTACAATAGGAAAAATAGGAAATATGAGACAAGCACATTGTAACATT  6780
319 GlyArgAlaPheValThrIleGlyLysIleGlyAsnMetArgGlnAlaHisCysAsnIle

    AGTAGAGCAAAATGGAATGCCACTTTAAAACAGATAGCTAGCAAATTAAGAGAACAATTT  6840
339 SerArgAlaLysTrpAsnAlaThrLeuLysGlnIleAlaSerLysLeuArgGluGlnPhe

    GGAAATAATAAAACAATAATCTTTAAGCAATCCTCAGGAGGGGACCCAGAAATTGTAACG  6900
359 GlyAsnAsnLysThrIleIlePheLysGlnSerSerGlyGlyAspProGluIleValThr

    CACAGTTTTAATTGTGGAGGGGAATTTTTCTACTGTAATTCAACACAACTGTTTAATAGT  6960
379 HisSerPheAsnCysGlyGlyGluPhePheTyrCysAsnSerThrGlnLeuPheAsnSer

    ACTTGGTTTAATAGTACTTGGAGTACTGAAGGGTCAAATAACACTGAAGGAAGTGACACA  7020
399 ThrTrpPheAsnSerThrTrpSerThrGluGlySerAsnAsnThrGluGlySerAspThr

    ATCACACTCCCATGCAGAATAAAACAATTTATAAACATGTGGCAGGAAGTAGGAAAAGCA  7080
419 IleThrLeuProCysArgIleLysGlnPheIleAsnMetTrpGlnGluValGlyLysAla

    ATGTATGCCCCTCCCATCAGCGGACAAATTAGATGTTCATCAAATATTACAGGGCTGCTA  7140
439 MetTyrAlaProProIleSerGlyGlnIleArgCysSerSerAsnIleThrGlyLeuLeu

    TTAACAAGAGATGGTGGTAATAACAACAATGGGTCCGAGATCTTCAGACCTGGAGGAGGA  7200
459 LeuThrArgAspGlyGlyAsnAsnAsnAsnGlySerGluIlePheArgProGlyGlyGly

    GATATGAGGGACAATTGGAGAAGTGAATTATATAAATATAAAGTAGTAAAAATTGAACCA  7260
479 AspMetArgAspAsnTrpArgSerGluLeuTyrLysTyrLysValValLysIleGluPro

    TTAGGAGTAGCACCCACCAAGGCAAAGAGAAGAGTGGTGCAGAGAGAAAAAAGAGCAGTG  7320
499 LeuGlyValAlaProThrLysAlaLysArgArgValValGlnArgGluLysArgAlaVal

    GGAATAGGAGCTTTGTTCCTTGGGTTCTTGGGAGCAGCAGGAAGCACTATGGGCGCACGG  7380
519 GlyIleGlyAlaLeuPheLeuGlyPheLeuGlyAlaAlaGlySerThrMetGlyAlaArg

    TCAATGACGCTGACGGTACAGGCCAGACAATTATTGTCTGGTATAGTGCAGCAGCAGAAC  7440
539 SerMetThrLeuThrValGlnAlaArgGlnLeuLeuSerGlyIleValGlnGlnGlnAsn

    AATTTGCTGAGGGCTATTGAGGCGCAACAGCATCTGTTGCAACTCACAGTCTGGGGCATC  7500
559 AsnLeuLeuArgAlaIleGluAlaGlnGlnHisLeuLeuGlnLeuThrValTrpGlyIle
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FIG. 2 (con't)

AAGCAGCTCCAGGCAAGAATCCTGGCTGTGGAAAGATACCTAAAGGATCAACAGCTCCTG 7560
579 LysGlnLeuGlnAlaArgIleLeuAlaValGluArgTyrLeuLysAspGlnGlnLeuLeu

GGGATTTGGGGTTGCTCTGGAAAACTCATTTGCACCACTGCTGTGCCTTGGAATGCTAGT 7620
599 GlyIleTrpGlyCysSerGlyLysLeuIleCysThrThrAlaValProTrpAsnAlaSer

TGGAGTAATAAATCTCTGGAACAGATTTGGAATAACATGACCTGGATGGAGTGGGACAGA 7680
619 TrpSerAsnLysSerLeuGluGlnIleTrpAsnAsnMetThrTrpMetGluTrpAspArg

GAAATTAACAATTACACAAGCTTAATACATTCCTTAATTGAAGAATCGCAAAACCAGCAA 7740
639 GluIleAsnAsnTyrThrSerLeuIleHisSerLeuIleGluGluSerGlnAsnGlnGln

GAAAAGAATGAACAAGAATTATTGGAATTAGATAAATGGGCAAGTTTGTGGAATTGGTTT 7800
659 GluLysAsnGluGlnGluLeuLeuGluLeuAspLysTrpAlaSerLeuTrpAsnTrpPhe

AACATAACAAATTGGCTGTGGTATATAAAAATATTCATAATGATAGTAGGAGGCTTGGTA 7860
679 AsnIleThrAsnTrpLeuTrpTyrIleLysIlePheIleMetIleValGlyGlyLeuVal

GGTTTAAGAATAGTTTTTGCTGTACTTTCTATAGTGAATAGAGTTAGGCAGGGATATTCA 7920
699 GlyLeuArgIleValPheAlaValLeuSerIleValAsnArgValArgGlnGlyTyrSer

CCATTATCGTTTCAGACCCACCTCCCAACCCCGAGGGGACCCGACAGGCCCGAAGGAATA 7980
719 ProLeuSerPheGlnThrHisLeuProThrProArgGlyProAspArgProGluGlyIle

GAAGAAGAAGGTGGAGAGAGAGACAGAGACAGATCCATTCGATTAGTGAACGGATCCTTA 8040
739 GluGluGluGlyGlyGluArgAspArgAspArgSerIleArgLeuValAsnGlySerLeu

GCACTTATCTGGGACGATCTGCGGAGCCTGTGCCTCTTCAGCTACCACCGCTTGAGAGAC 8100
759 AlaLeuIleTrpAspAspLeuArgSerLeuCysLeuPheSerTyrHisArgLeuArgAsp

TTACTCTTGATTGTAACGAGGATTGTGGAACTTCTGGGACGCAGGGGGTGGGAAGCCCTC 8160
779 LeuLeuLeuIleValThrArgIleValGluLeuLeuGlyArgArgGlyTrpGluAlaLeu

AAATATTGGTGGAATCTCCTACAGTATTGGAGTCAGGAACTAAAGAATAGTGCTGTTAGC 8220
799 LysTyrTrpTrpAsnLeuLeuGlnTyrTrpSerGlnGluLeuLysAsnSerAlaValSer

TTGCTCAATGCCACAGCCATAGCAGTAGCTGAGGGGACAGATAGGGTTATAGAAGTAGTA 8280
819 LeuLeuAsnAlaThrAlaIleAlaValAlaGluGlyThrAspArgValIleGluValVal

CAAGGAGCTTGTAGAGCTATTCGCCACATACCTAGAAGAATAAGACAGGGCTTGGAAAGG 8340
839 GlnGlyAlaCysArgAlaIleArgHisIleProArgArgIleArgGlnGlyLeuGluArg

ATTTTGCTATAAGATGGGTGGCAAGTGGTCAAAAAGTAGTGTGGTTGGATGGCCTACTGT 8400
859 IleLeuLeu***

AAGGGAAAGAATGAGACGAGCTGAGCCAGCAGCAGATGGGGTGGGAGCAGCATCTCGAGA 8460

CCTGGAAAAACATGGAGCAATCACAAGTAGCAATACAGCAGCTACCAATGCTGCTTGTGC 8520

KpnI
CTGGCTAGAAGCACAAGAGGAGGAGGAGGTGGGTTTTCCAGTCACACCTCAGGTACCTTT 8580

AAGACCAATGACTTACAAGGCAGCTCTAGATCTTAGCCACTTTTTAAAAGAAAAGGGGGG 8640

FIG. 2 (cont)

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ACTGGAAGGGCTAATTCACTCCCAACGAAGACAAGATATCCTTGATCTGTGGATCTACCA   8700
CACACAAGGCTACTTCCCTGATTGGCAGAACTACACACCAGGGCCAGGGGTCAGATATCC   8760
ACTGACCTTTGGATGGTGCTACAAGCTAGTACCAGTTGAGCCAGATAAGGTAGAAGAGGC   8820
CAATAAAGGAGAGAACACCAGCTTGTTACACCCTGTGAGCCTGCATGGAATGGATGACCC   8880
TGAGAGAGAAGTGTTAGAGTGGAGGTTTGACAGCCGCCTACGATTTCATCACGTGGCCCG   8940
AGAGCTGCATCCGGAGTACTTCAAGAACTGCTGACATCGAGCTTGCTACAAGGGACTTTC   9000
CGCTGGGGACTTTCCAGGGAGGCGTGGCCTGGGCGGGACTGGGGAGTGGCGAGCCCTCAG   9060
ATGCTGCATATAAGCAGCTGCTTTTTGCCTGTACTGGGTCTCTCTGGTTAGACCAGATTT   9120
           SstI
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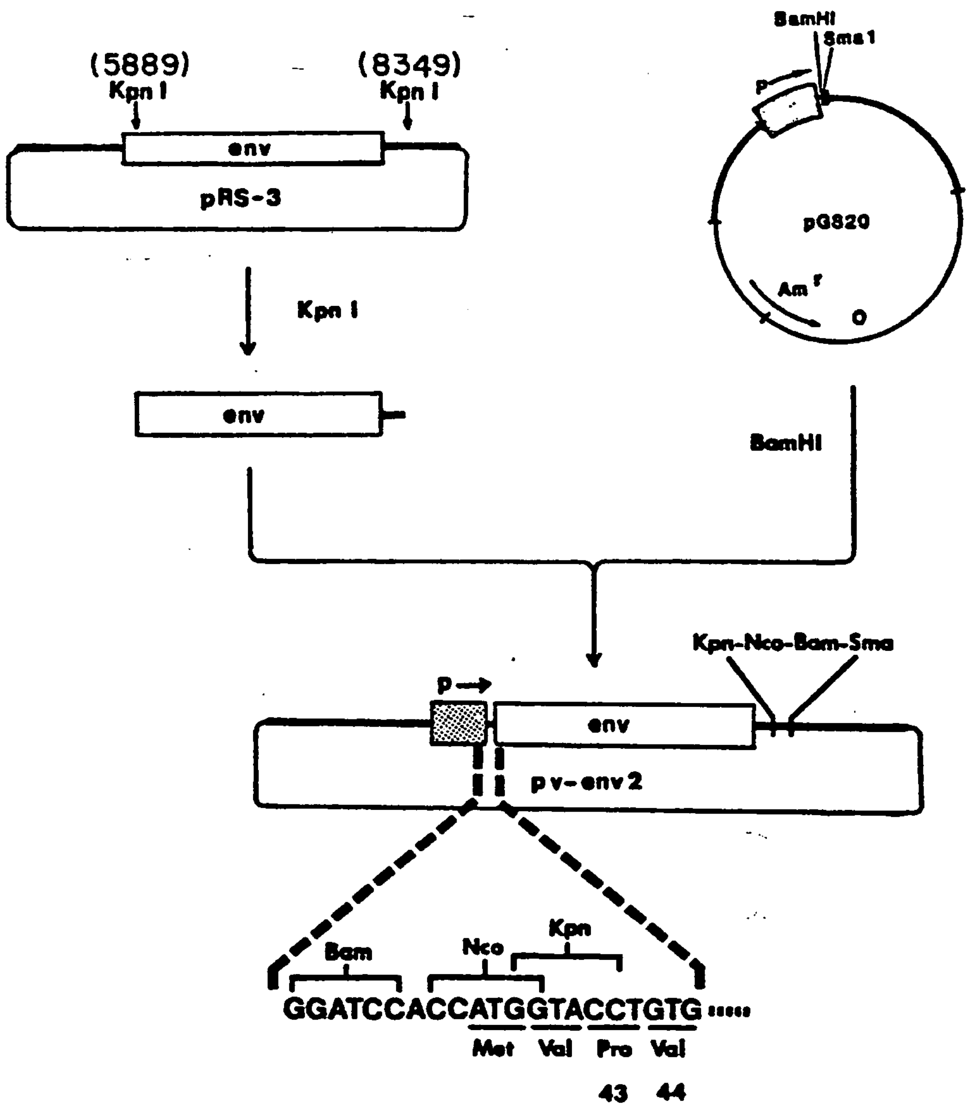
FIG. 3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(8349)
Kpn I
env
pRS-3
Kpn I
env
BamHI
Sma1
P
pG820
Am r
O
BamHI
Kpn-Nco-Bam-Sma
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env
pv-env2
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Nco
Kpn
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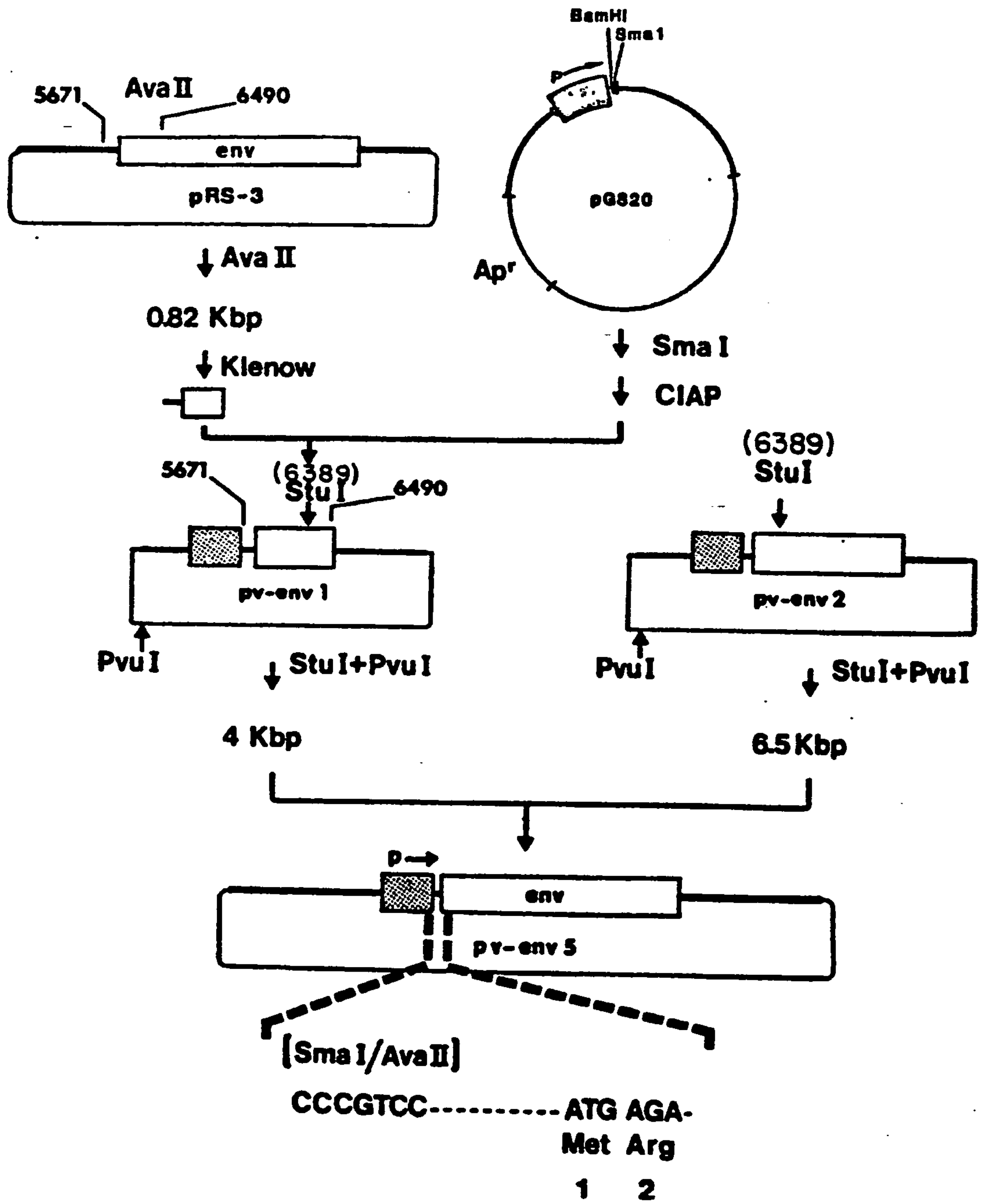
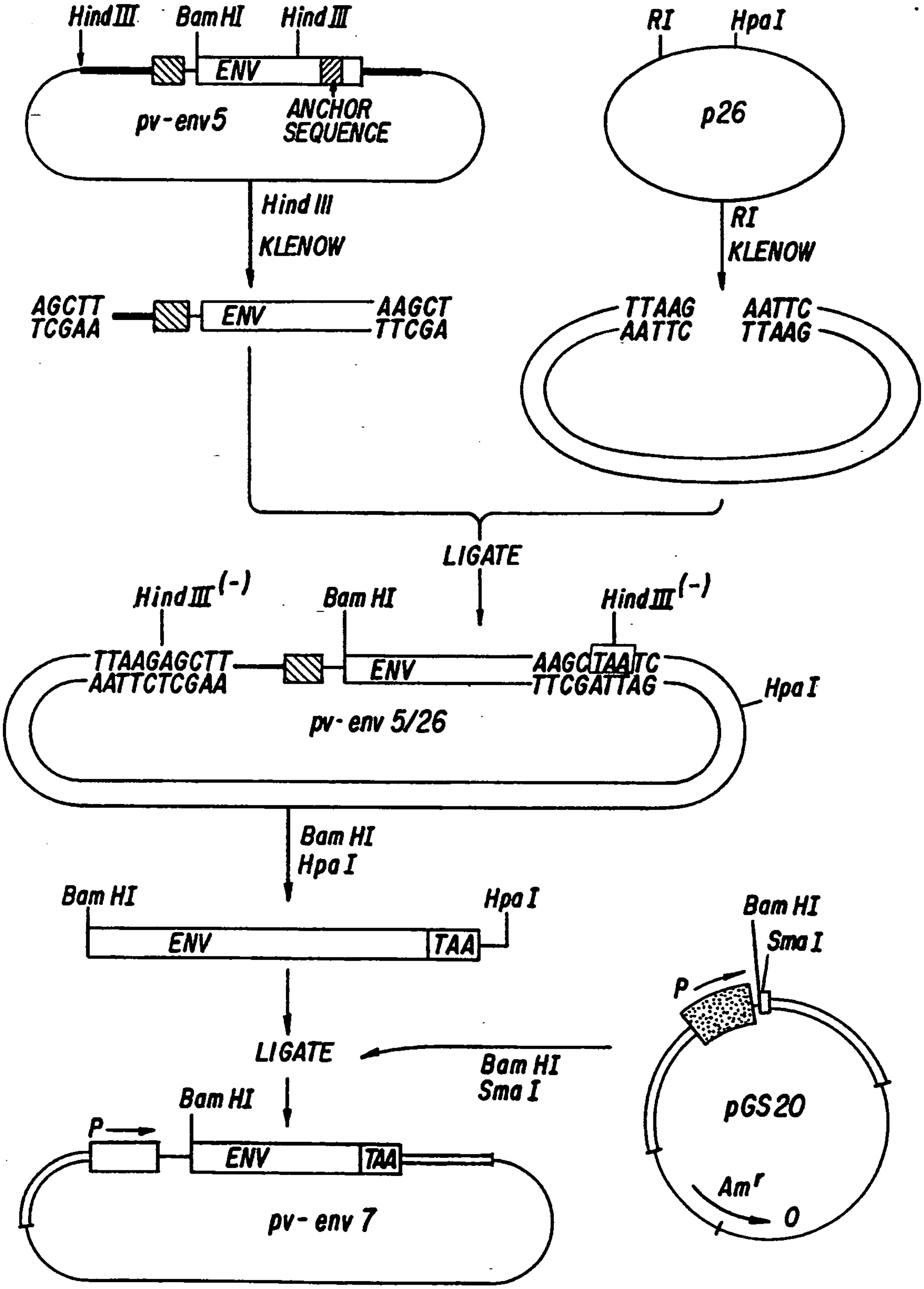
Met Val Pro Val
43 44

FIG. 4



SUBSTITUTE SHEET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BamHI
HindIII
ENV
pv-env5
ANCHOR SEQUENCE
RI
HpaI
p26
Hind III
KLENOW
RI
KLENOW
AGCTT
TCGAA
ENV
AAGCT
TTCGA
TTAAG
AATTC
AATTC
TTAAG
LIGATE
HindIII(-)
BamHI
HindIII(-)
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AATTCTCGAA
ENV
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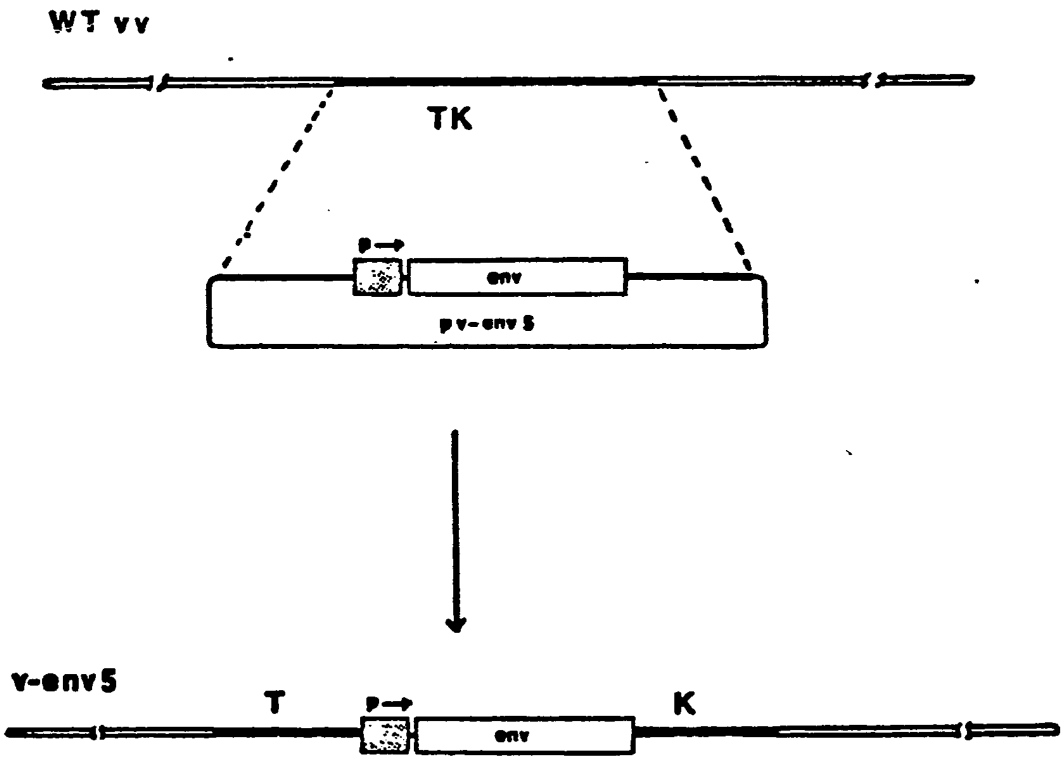
TTCGATTAG
HpaI
pv-env 5/26
BamHI
HpaI
BamHI
HpaI
ENV
TAA
BamHI
SmaI
P
pGS20
Amr
O
LIGATE
BamHI
SmaI
BamHI
P
ENV
TAA
pv-env 7

FIG. 5

FIG. 6



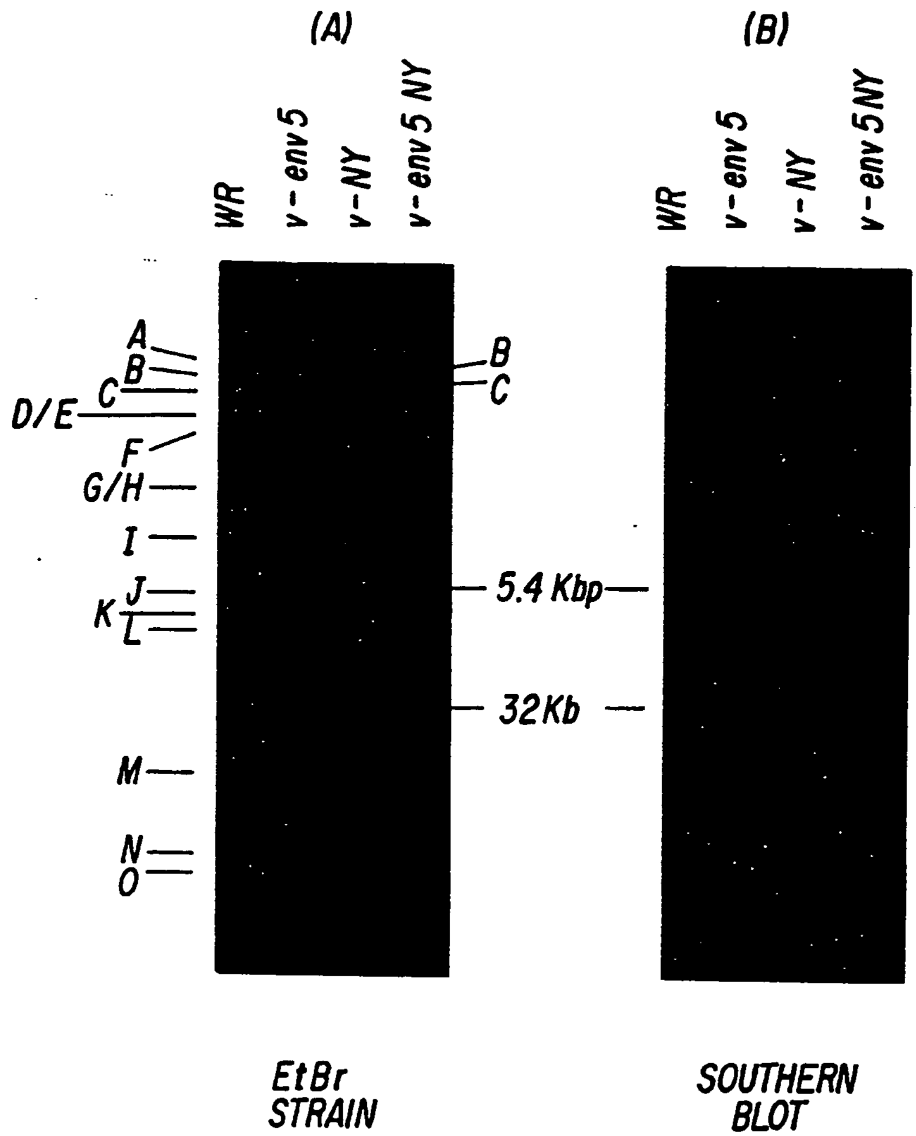

(A)
(B)
WR
v-env 5
v-NY
v-env 5 NY
A
B
C
D/E
F
G/H
I
J
K
L
M
N
O
B
C
5.4 Kbp
32Kb
EtBr STRAIN
SOUTHERN BLOT

FIG. 7

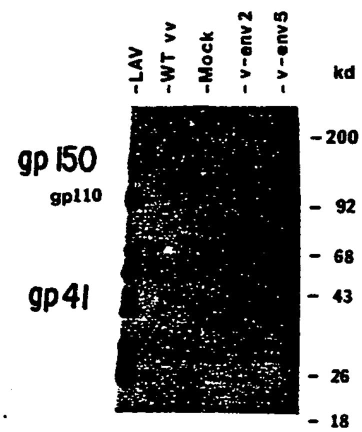

-LAV
-WT vv
-Mock
-v-env2
-v-env5
kd
gp150
gp110
gp41
-200
-92
-68
-43
-26
-18

FIG. 8A

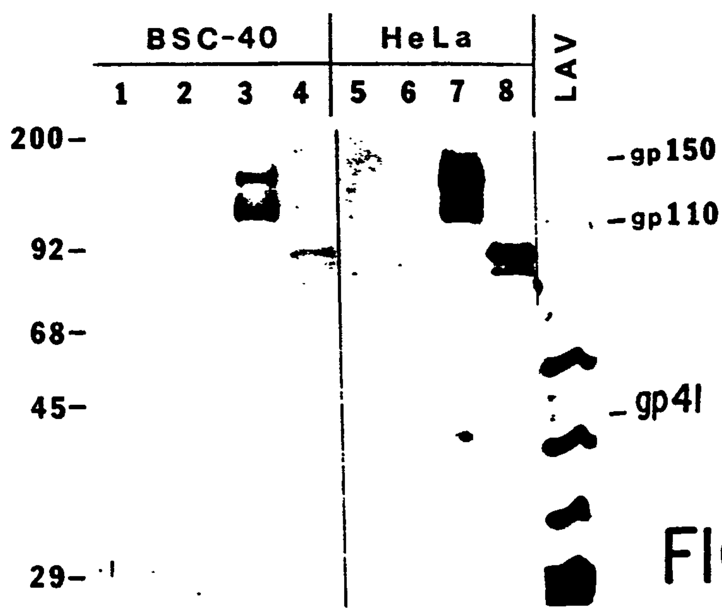
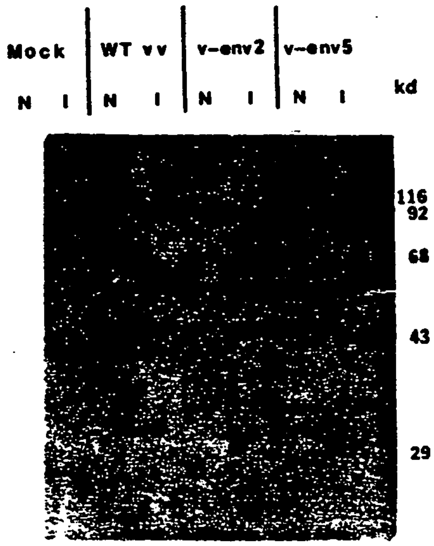
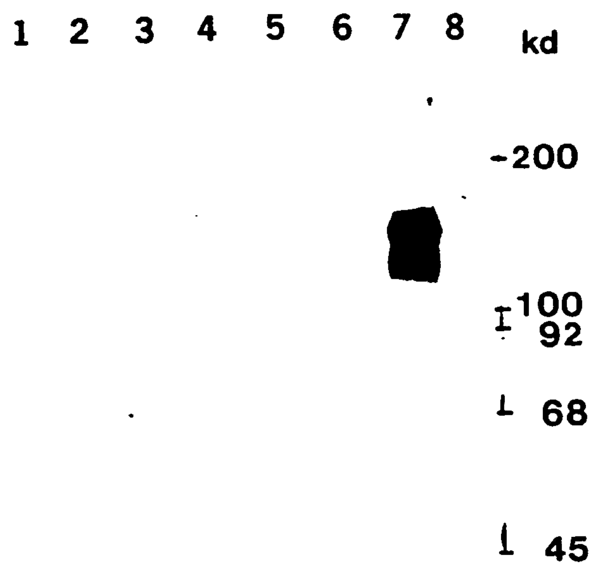

BSC-40
HeLa
1 2 3 4 5 6 7 8
LAV
200-
92-
68-
45-
29-
-gp150
-gp110
-gp41

FIG. 8B



Mock
WT vv
v-env2
v-env5
N I N I N I N I
kd
116
92
68
43
29

FIG. 9A



1 2 3 4 5 6 7 8
kd
200
100
92
68
45

FIG. 9B

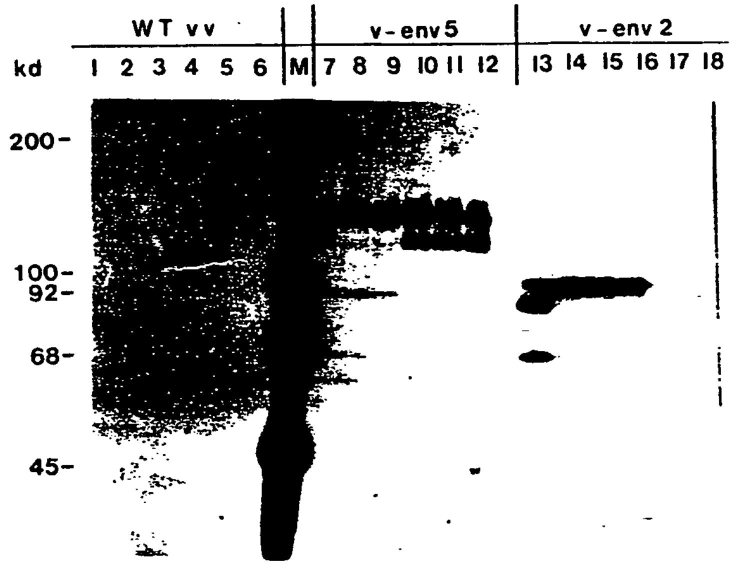

WT vv
v-env5
v-env2
kd
1 2 3 4 5 6 M 7 8 9 10 11 12 13 14 15 16 17 18
200-
100-
92-
68-
45-

FIG. 9C

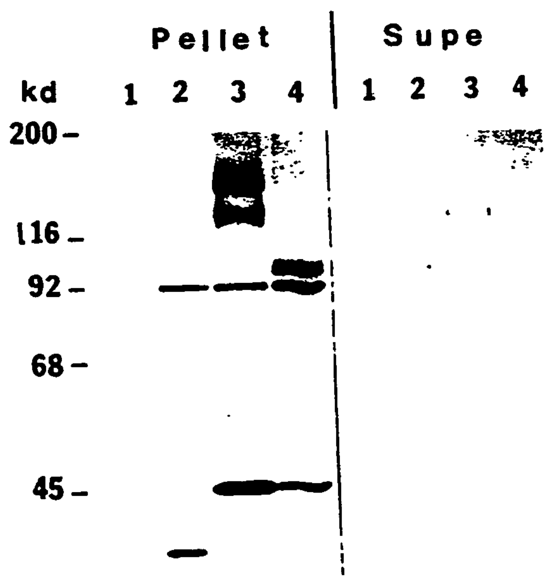

Pellet
Supe
kd
1 2 3 4
1 2 3 4
200-
116-
92-
68-
45-

FIG. 9D

FIG. 9E

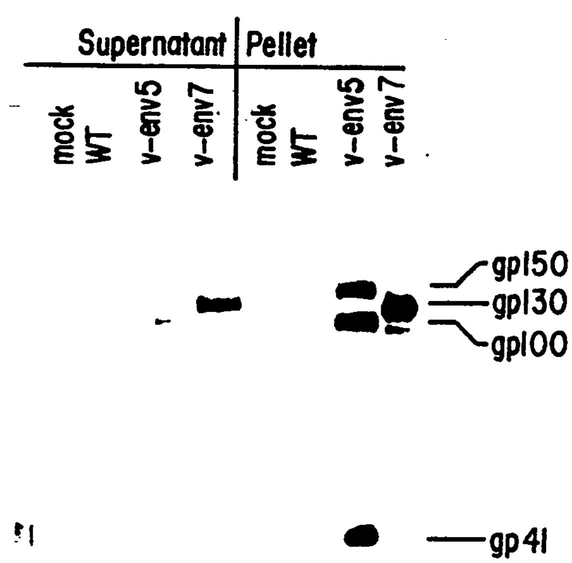
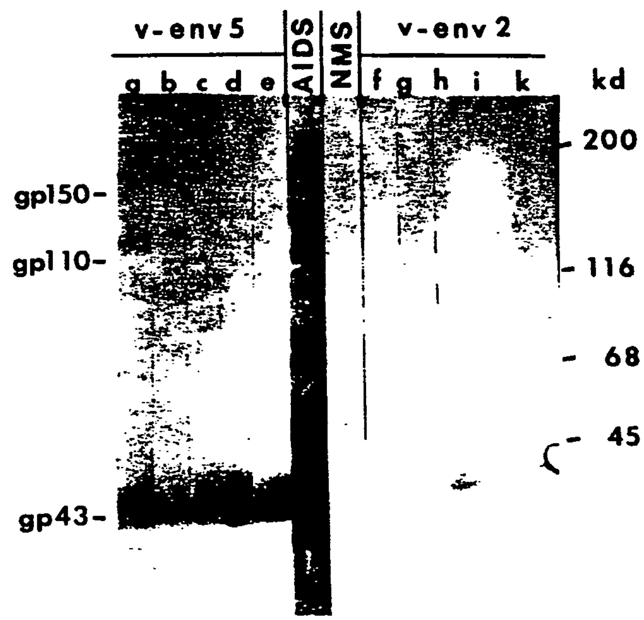

Supernatant
Pellet
mock
WT
v-env5
v-env7
mock
WT
v-env5
v-env7
gp150
gp130
gp100
gp41

FIG. 10


v-env 5
AIDS
NMS
v-env 2
a b c d e
f g h i k
kd
200
116
68
45
gp150-
gp110-
gp43-

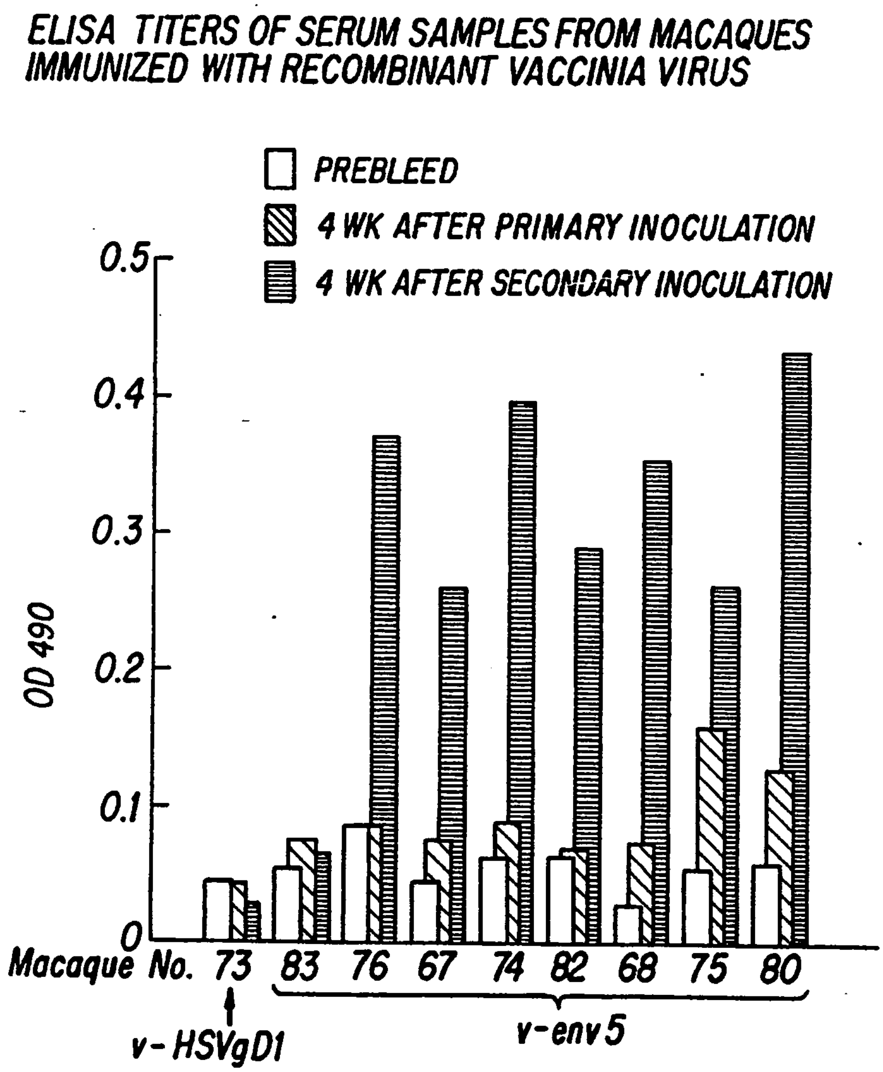

ELISA TITERS OF SERUM SAMPLES FROM MACAQUES IMMUNIZED WITH RECOMBINANT VACCINIA VIRUS
PREBLEED
4 WK AFTER PRIMARY INOCULATION
4 WK AFTER SECONDARY INOCULATION
0.5
0.4
0.3
0.2
0.1
0
OD 490
Macaque No.
73
83
76
67
74
82
68
75
80
v-HSVgD1
v-env5

FIG. 11

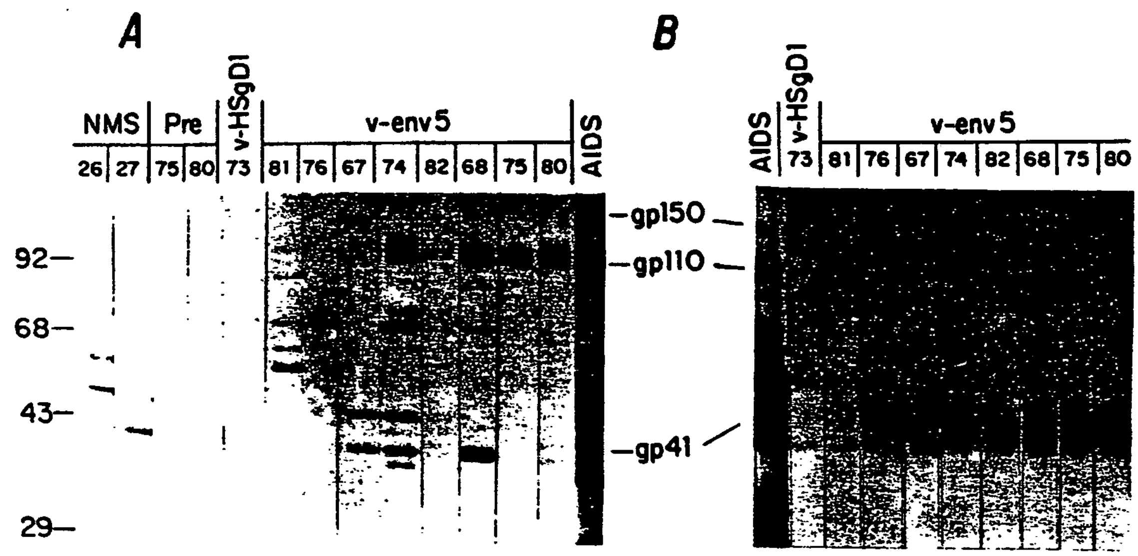
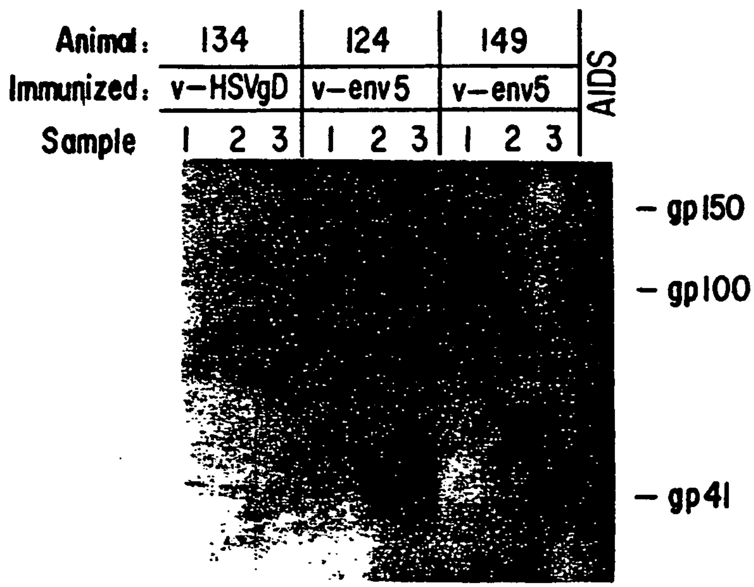

A
B
NMS
Pre
v-HSgD1
v-env5
AIDS
26
27
75
80
73
81
76
67
74
82
68
75
80
92—
68—
43—
29—
—gp150
—gp110
—gp41
AIDS
v-HSgD1
v-env5
73
81
76
67
74
82
68
75
80

FIG.12

FIG. 13



Sample 1 = Prebleed
Sample 2 = 8 wk after 1st immunization
Sample 3 = 2 wk after 2nd immunization

FIG. 14

GGTCTCTCTGGTTAGACCAGATTTGAGCCTGGGAGCTCTCTGGCTAACTAGGGAACCCAC 60

TGCTTAAGCCTCAATAAAGCTTGCCTTGAGTGCTTCAAGTAGTGTGTGCCCGTCTGTTGT 120

GTGACTCTGGTAACTAGAGATCCCTCAGACCCTTTTAGTCAGTGTGGAAAATCTCTAGCA 180

pAc-gag1
SstI
GTGGCGCCCGAACAGGGACTTGAAAGCGAAAGGGAAACCAGAGGAGCTCTCTCGACGCAG 240
start of pKS-5

pvgag1,2,3,4,5
ThaI
GACTCGGCTTGCTGAAGCGCGCACGGCAAGAGGCGAGGGGCGGCGACTGGTGAGTACGCC 300

GAG→
AAAAATTTTGACTAGCGGAGGCTAGAAGGAGAGAGATGGGTGCGAGAGCGTCAGTATTAA 360

-11 ***LeuAlaGluAlaArgArgArgGluMetGlyAlaArgAlaSerValLeuSer

GCGGGGGAGAATTAGATCGATGGGAAAAAATTCGGTTAAGGCCAGGGGGAAAGAAAAAAT 420

10 GlyGlyGluLeuAspArgTrpGluLysIleArgLeuArgProGlyGlyLysLysLysTyr

ATAAATTAAAACATATAGTATGGGCAAGCAGGGAGCTAGAACGATTCGCAGTTAATCCTG 480

30 LysLeuLysHisIleValTrpAlaSerArgGluLeuGluArgPheAlaValAsnProGly

GCCTGTTAGAAACATCAGAAGGCTGTAGACAAATACTGGGACAGCTACAACCATCCCTTC 540

50 LeuLeuGluThrSerGluGlyCysArgGlnIleLeuGlyGlnLeuGlnProSerLeuGln

AGACAGGATCAGAAGAACTTAGATCATTATATAATACAGTAGCAACCCTCTATTGTGTGC 600

70 ThrGlySerGluGluLeuArgSerLeuTyrAsnThrValAlaThrLeuTyrCysValHis

ATCAAAGGATAGAGATAAAAGACACCAAGGAAGCTTTAGACAAGATAGAGGAAGAGCAAA 660

90 GlnArgIleGluIleLysAspThrLysGluAlaLeuAspLysIleGluGluGluGlnAsn

FIG. 14 (con't)

ACAAAAGTAAGAAAAAAGCACAGCAAGCAGCAGCTGACACAGGACACAGCAGCCAGGTCA 720

110 LysSerLysLysLysAlaGlnGlnAlaAlaAlaAspThrGlyHisSerSerGlnValSer

GCCAAAATTACCCTATAGTGCAGAACATCCAGGGGCAAATGGTACATCAGGCCATATCAC 780

130 GlnAsnTyrProIleValGlnAsnIleGlnGlyGlnMetValHisGlnAlaIleSerPro

CTAGAACTTTAAATGCATGGGTAAAAGTAGTAGAAGAGAAGGCTTTCAGCCCAGAAGTGA 840

150 ArgThrLeuAsnAlaTrpValLysValValGluGluLysAlaPheSerProGluValIle

TACCCATGTTTTCAGCATTATCAGAAGGAGCCACCCCACAAGATTTAAACACCATGCTAA 900

170 ProMetPheSerAlaLeuSerGluGlyAlaThrProGlnAspLeuAsnThrMetLeuAsn

ACACAGTGGGGGGACATCAAGCAGCCATGCAAATGTTAAAAGAGACCATCAATGAGGAAG 960

190 ThrValGlyGlyHisGlnAlaAlaMetGlnMetLeuLysGluThrIleAsnGluGluAla

CTGCAGAATGGGATAGAGTGCATCCAGTGCATGCAGGGCCTATTGCACCAGGCCAGATGA 1020

210 AlaGluTrpAspArgValHisProValHisAlaGlyProIleAlaProGlyGlnMetArg

GAGAACCAAGAGGAAGTGACATAGCAGGAACTACTAGTACCCTTCAGGAACAAATAGGAT 1080

230 GluProArgGlySerAspIleAlaGlyThrThrSerThrLeuGlnGluGlnIleGlyTrp

GGATGACAAATAATCCACCTATCCCAGTAGGAGAAATTTATAAAAGATGGATAATCCTGG 1140

250 MetThrAsnAsnProProIleProValGlyGluIleTyrLysArgTrpIleIleLeuGly

GATTAAATAAAATAGTAAGAATGTATAGCCCTACCAGCATTCTGGACATAAGACAAGGAC 1200

270 LeuAsnLysIleValArgMetTyrSerProThrSerIleLeuAspIleArgGlnGlyPro

CAAAAGAACCCTTTAGAGACTATGTAGACCGGTTCTATAAAACTCTAAGAGCCGAGCAAG 1260

290 LysGluProPheArgAspTyrValAspArgPheTyrLysThrLeuArgAlaGluGlnAla

CTTCACAGGAGGTAAAAAATTGGATGACAGAAACCTTGTTGGTCCAAAATGCGAACCCAG 1320

310 SerGlnGluValLysAsnTrpMetThrGluThrLeuLeuValGlnAsnAlaAsnProAsp

FIG. 14 (con't)

```
     ATTGTAAGACTATTTTAAAAGCATTGGGACCAGCAGCTACACTAGAAGAAATGATGACAG    1380

330   CysLysThrIleLeuLysAlaLeuGlyProAlaAlaThrLeuGluGluMetMetThrAla
     CATGTCAGGGAGTGGGAGGACCCGGCCATAAGGCAAGAGTTTTGGCTGAAGCAATGAGCC    1440

350   CysGlnGlyValGlyGlyProGlyHisLysAlaArgValLeuAlaGluAlaMetSerGln
     AAGTAACAAATTCAGCTACCATAATGATGCAAAGAGGCAATTTTAGGAACCAAAGAAAGA    1500

370   ValThrAsnSerAlaThrIleMetMetGlnArgGlyAsnPheArgAsnGlnArgLysIle
     TTGTTAAGTGTTTCAATTGTGGCAAAGAAGGGCACATAGCCAGAAATTGCAGGGCCCCTA    1560

390   ValLysCysPheAsnCysGlyLysGluGlyHisIleAlaArgAsnCysArgAlaProArg
     GGAAAAAGGGCTGTTGGAAATGTGGAAAGGAAGGACACCAAATGAAAGATTGTACTGAGA    1620

410   LysLysGlyCysTrpLysCysGlyLysGluGlyHisGlnMetLysAspCysThrGluArg
                              Bgl II
     GACAGGCTAATTTTTTAGGGAAGATCTGGCCTTCCTACAAGGGAAGGCCAGGGAATTTTC    1680
            TPb →
            ***PhePheArgGluAspLeuAlaPheLeuGlnGlyLysAlaArgGluPheSer
430   GlnAlaAsnPheLeuGlyLysIleTrpProSerTyrLysGlyArgProGlyAsnPheLeu

     TTCAGAGCAGACCAGAGCCAACAGCCCCACCAGAAGAGAGCTTCAGGTCTGGGGTAGAGA    1740

      SerGluGlnThrArgAlaAsnSerProThrArgArgGluLeuGlnValTrpGlyArgAsp
450    GlnSerArgProGluProThrAlaProProGluGluSerPheArgSerGlyValGluThr

     CAACAACTCCCTCTCAGAAGCAGGAGCCGATAGACAAGGAACTGTATCCTTTAACTTCCC    1800

      AsnAsnSerLeuSerGluAlaGlyAlaAspArgGlnGlyThrValSerPheAsnPhePro
470    ThrThrProSerGlnLysGlnGluProIleAspLysGluLeuTyrProLeuThrSerLeu

     TCAGATCACTCTTTGGCAACGACCCCTCGTCACAATAAAGATAGGGGGGCAACTAAAGGA    1860

      GlnIleThrLeuTrpGlnArgProLeuValThrIleLysIleGlyGlyGlnLeuLysGlu
490    ArgSerLeuPheGlyAsnAspProSerSerGln***
                                        STOP
     AGCTCTATTAGATACAGGAGCAGATGATACAGTATTAGAAGAAATGAGTTTGCCAGGAAG    1920

      AlaLeuLeuAspThrGlyAlaAspAspThrValLeuGluGluMetSerLeuProGlyArg
                                                             Bcl I
     ATGGAAACCAAAAATGATAGGGGGAATTGGAGGTTTTATCAAAGTAAGACAGTATGATCA    1980

      TrpLysProLysMetIleGlyGlyIleGlyGlyPheIleLysValArgGlnTyrAspGln
```

FIG.14 (con't)

GATACTCATAGAAATCTGTGGACATAAAGCTATAGGTACAGTATTAGTAGGACCTACACC 2040

IleLeuIleGluIleCysGlyHisLysAlaIleGlyThrValLeuValGlyProThrPro

HincII

TGTCAACATAATTGGAAGAAATCTGTTGACTCAGATTGGTTGCACTTTAAATTTTCCCAT 2100

ValAsnIleIleGlyArgAsnLeuLeuThrGlnIleGlyCysThrLeuAsnPheProIle

TAGTCCTATTGAAACTGTACCAGTAAAATTAAAGCCAGGAATGGATGGCCCAAAAGTTAA 2160

SerProIleGluThrValProValLysLeuLysProGlyMetAspGlyProLysValLys

ACAATGGCCATTGACAGAAGAAAAAATAAAAGCATTAGTAGAAATTTGTACAGAAATGGA 2220

GlnTrpProLeuThrGluGluLysIleLysAlaLeuValGluIleCysThrGluMetGlu

AAAGGAAGGGAAAATTTCAAAAATTGGGCCTGAAAATCCATACAATACTCCAGTATTTGC 2280

LysGluGlyLysIleSerLysIleGlyProGluAsnProTyrAsnThrProValPheAla

CATAAAGAAAAAAGACAGTACTAAATGGAGAAAATTAGTAGATTTCAGAGAACTTAATAA 2340

IleLysLysLysAspSerThrLysTrpArgLysLeuValAspPheArgGluLeuAsnLys

GAGAACTCAAGACTTCTGGGAAGTTCAATTAGGAATACCACATCCCGCAGGGTTAAAAAA 2400

ArgThrGlnAspPheTrpGluValGlnLeuGlyIleProHisProAlaGlyLeuLysLys

GAAAAAATCAGTAACAGTACTGGATGTGGGTGATGCATATTTTTCAGTTCCCTTAGATGA 2460

LysLysSerValThrValLeuAspValGlyAspAlaTyrPheSerValProLeuAspGlu

AGACTTCAGGAAGTATACTGCATTTACCATACCTAGTATAAACAATGAGACACCAGGGAT 2520

AspPheArgLysTyrThrAlaPheThrIleProSerIleAsnAsnGluThrProGlyIle

EcoRV

TAGATATCAGTACAATGTGCTTCCACAGGGATGGAAAGGATCACCAGCAATATTCCAAAG 2580

ArgTyrGlnTyrAsnValLeuProGlnGlyTrpLysGlySerProAlaIlePheGlnSer

TAGCATGACAAAAATCTTAGAGCCTTTTAGAAAACAAAATCCAGACATAGTTATCTATCA 2640

SerMetThrLysIleLeuGluProPheArgLysGlnAsnProAspIleValIleTyrGln

FIG. 14 (con't)

ATACATGGATGATTTGTATGTAGGATCTGACTTAGAAATAGGGCAGCATAGAACAAAAAT 2700
TyrMetAspAspLeuTyrValGlySerAspLeuGluIleGlyGlnHisArgThrLysIle

AGAGGAGCTGAGACAACATCTGTTGAGGTGGGGACTTACCACACCAGACAAAAAACATCA 2760
GluGluLeuArgGlnHisLeuLeuArgTrpGlyLeuThrThrProAspLysLysHisGln

GAAAGAACCTCCATTCCTTTGGATGGGTTATGAACTCCATCCTGATAAATGGACAGTACA 2820
LysGluProProPheLeuTrpMetGlyTyrGluLeuHisProAspLysTrpThrValGln

GCCTATAGTGCTGCCAGAAAAAGACAGCTGGACTGTCAATGACATACAGAAGTTAGTGGG 2880
ProIleValLeuProGluLysAspSerTrpThrValAsnAspIleGlnLysLeuValGly

AAAATTGAATTGGGCAAGTCAGATTTACCCAGGGATTAAAGTAAGGCAATTATGTAAACT 2940
LysLeuAsnTrpAlaSerGlnIleTyrProGlyIleLysValArgGlnLeuCysLysLeu

CCTTAGAGGAACCAAAGCACTAACAGAAGTAATACCACTAACAGAAGAAGCAGAGCTAGA 3000
LeuArgGlyThrLysAlaLeuThrGluValIleProLeuThrGluGluAlaGluLeuGlu

ACTGGCAGAAAACAGAGAGATTCTAAAAGAACCAGTACATGGAGTGTATTATGACCCATC 3060
LeuAlaGluAsnArgGluIleLeuLysGluProValHisGlyValTyrTyrAspProSer

AAAAGACTTAATAGCAGAAATACAGAAGCAGGGGCAAGGCCAATGGACATATCAAATTTA 3120
LysAspLeuIleAlaGluIleGlnLysGlnGlyGlnGlyGlnTrpThrTyrGlnIleTyr

TCAAGAGCCATTTAAAAATCTGAAAACAGGAAAATATGCAAGAACGAGGGGTGCCCACAC 3180
GlnGluProPheLysAsnLeuLysThrGlyLysTyrAlaArgThrArgGlyAlaHisThr

TAATGATGTAAAACAATTAACAGAGGCAGTGCAAAAAATAACCACAGAAAGCATAGTAAT 3240
AsnAspValLysGlnLeuThrGluAlaValGlnLysIleThrThrGluSerIleValIle

ATGGGGAAAGACTCCTAAATTTAAACTACCCATACAAAAGGAAACATGGGAAACATGGTG 3300
TrpGlyLysThrProLysPheLysLeuProIleGlnLysGluThrTrpGluThrTrpTrp

FIG. 14 (con't)

GACAGAGTATTGGCAAGCCACCTGGATTCCTGAGTGGGAGTTTGTCAATACCCCTCCTTT 3360

ThrGluTyrTrpGlnAlaThrTrpIleProGluTrpGluPheValAsnThrProProLeu

KpnI

AGTGAAATTATGGTACCAGTTAGAGAAAGAACCCATAGTAGGAGCAGAAACGTTCTATGT 3420

End of pKS-5

ValLysLeuTrpTyrGlnLeuGluLysGluProIleValGlyAlaGluThrPheTyrVal

AGATGGGGCAGCTAGCAGGGAGACTAAATTAGGAAAAGCAGGATATGTTACTAATAGAGG 3480

AsnGluAlaAlaSerArgGluThrLysLeuGlyLysAlaGlyTyrValThrAsnArgGly

AAGACAAAAAGTTGTCACCCTAACTGACACAACAAATCAGAAGACTGAGTTACAAGCAAT 3540

ArgGlnLysValValThrLeuThrAspThrThrAsnGlnLysThrGluLeuGlnAlaIle

TCATCTAGCTTTGCAGGATTCGGGATTAGAAGTAAATATAGTAACAGACTCACAATATGC. 3600

HisLeuAlaLeuGlnAspSerGlyLeuGluValAsnIleValThrAspSerGlnTyrAla

ATTAGGAATCATTCAAGCACAACCAGATAAAAGTGAATCAGAGTTAGTCAATCAAATAAT 3660

LeuGlyIleIleGlnAlaGlnProAspLysSerGluSerGluLeuValAsnGlnIleIle

AGAGCAGTTAATAAAAAAGGAAAAGGTCTATCTGGCATGGGTACCAGCACACAAAGGAAT 3720

GluGlnLeuIleLysLysGluLysValTyrLeuAlaTrpValProAlaHisLysGlyIle

TGGAGGAAATGAACAAGTAGATAAATTAGTCAGTGCTGGAATCAGGAAAGTACTATTTTT 3780

GlyGlyAsnGluGlnValAspLysLeuValSerAlaGlyIleArgLysValLeuPheLeu

AGATGGAATAGATAAGGCCCAAGATGAACATGAGAAATATCACAGTAATTGGAGAGCAAT 3840

AspGlyIleAspLysAlaGlnAspGluHisGluLysTyrHisSerAsnTrpArgAlaMet

GGCTAGTGATTTTAACCTGCCACCTGTAGTAGCAAAAGAAATAGTAGCCAGCTGTGATAA 3900

AlaSerAspPheAsnLeuProProValValAlaLysGluIleValAlaSerCysAspLys

ATGTCAGCTAAAAGGAGAAGCCATGCATGGACAAGTAGACTGTAGTCCAGGAATATGGCA 3960

CysGlnLeuLysGlyGluAlaMetHisGlyGlnValAspCysSerProGlyIleTrpGln

FIG. 14 (con't)

ACTAGATTGTACACATTTAGAAGGAAAAGTTATCCTGGTAGCAGTTCATGTAGCCAGTGG 4020

LeuAspCysThrHisLeuGluGlyLysValIleLeuValAlaValHisValAlaSerGly

ATATATAGAAGCAGAAGTTATTCCAGCAGAAACAGGGCAGGAAACAGCATACTTTCTTTT 4080

TyrIleGluAlaGluValIleProAlaGluThrGlyGlnGluThrAlaTyrPheLeuLeu

AAAATTAGCAGGAAGATGGCCAGTAAAAACAATACATACAGACAATGGCAGCAATTTCAC 4140

LysLeuAlaGlyArgTrpProValLysThrIleHisThrAspAsnGlySerAsnPheThr

EcoRI
CAGTACTACGGTTAAGGCCGCCTGTTGGTGGGCGGGAATCAAGCAGGAATTTGGAATTCC 4200

SerThrThrValLysAlaAlaCysTrpTrpAlaGlyIleLysGlnGluPheGlyIlePro

CTACAATCCCCAAAGTCAAGGAGTAGTAGAATCTATGAATAAAGAATTAAAGAAAATTAT 4260

TyrAsnProGlnSerGlnGlyValValGluSerMetAsnLysGluLeuLysLysIleIle

AGGCCAGGTAAGAGATCAGGCTGAACATCTTAAGACAGCAGTACAAATGGCAGTATTCAT 4320

GlyGlnValArgAspGlnAlaGluHisLeuLysThrAlaValGlnMetAlaValPheIle

CCACAATTTTAAAAGAAAAGGGGGGATTGGGGGGTACAGTGCAGGGGAAAGAATAGTAGA 4380

HisAsnPheLysArgLysGlyGlyIleGlyGlyTyrSerAlaGlyGluArgIleValAsp

CATAATAGCAACAGACATACAAACTAAAGAATTACAAAAACAAATTACAAAAATTCAAAA 4440

IleIleAlaThrAspIleGlnThrLysGluLeuGlnLysGlnIleThrLysIleGlnAsn

TTTTCGGGTTTATTACAGGGACAGCAGAGATCCACTTTGGAAAGGACCAGCAAAGCTCCT 4500

PheArgValTyrTyrArgAspSerArgAspProLeuTrpLysGlyProAlaLysLeuLeu

CTGGAAAGGTGAAGGGGCAGTAGTAATACAAGATAATAGTGACATAAAAGTAGTGCCAAG 4560

TrpLysGlyGluGlyAlaValValIleGlnAspAsnSerAspIleLysValValProArg

AAGAAAAGCAAAGATCATTAGGGATTATGGAAAACAGATGGCAGGTGATGATTGTGTGGC 4620

ArgLysAlaLysIleIleArgAspTyrGlyLysGlnMetAlaGlyAspAspCysValAla

FIG. 14 (con't)

```
AAGTAGACAGGATGAGGATTAGAACATGGAAAAGTTTAGTAAAACACCATATGTATGTTT    4680
 SerArgGlnAspGluAsp*** End of Pol

CAGGGAAAGCTAGGGGATGGTTTTATAGACATCACTATGAAAGCCCTCATCCAAGAATAA    4740

GTTCAGAAGTACACATCCCACTAGGGGATGCTAGATTGGTAATAACAACATATTGGGGTC    4800

TGCATACAGGAGAAAGAGACTGGCATCTGGGTCAGGGAGTCTCCATAGAATGGAGGAAAA    4860

AGAGATATAGCACACAAGTAGACCCTGAACTAGCAGACCAACTAATTCATCTGTATTACT    4920

TTGACTGTTTTTCAGACTCTGCTATAAGAAAGGCCTTATTAGGACATATAGTTAGCCCTA    4980

GGTGTGAATATCAAGCAGGACATAACAAGGTAGGATCTCTACAATACTTGGCACTAGCAG    5040

CATTAATAACACCAAAAAAGATAAAGCCACCTTTGCCTAGTGTTACGAAACTGACAGAGG    5100

ATAGATGGAACAAGCCCCAGAAGACCAAGGGCCACAGAGGGAGCCACACAATGAATGGAC    5160

ACTAGAGCTTTTAGAGGAGCTTAAGAATGAAGCTGTTAGACATTTTCCTAGGATTTGGCT    5220

CCATGGCTTAGGGCAACATATCTATGAAACTTATGGGGATACTTGGGCAGGAGTGGAAGC    5280

CATAATAAGAATTCTGCAACAACTGCTGTTTATCCATTTCAGAATTGGGTGTCGACATAG    5340
                                                  SalI
                                                  end of pSS.5
```

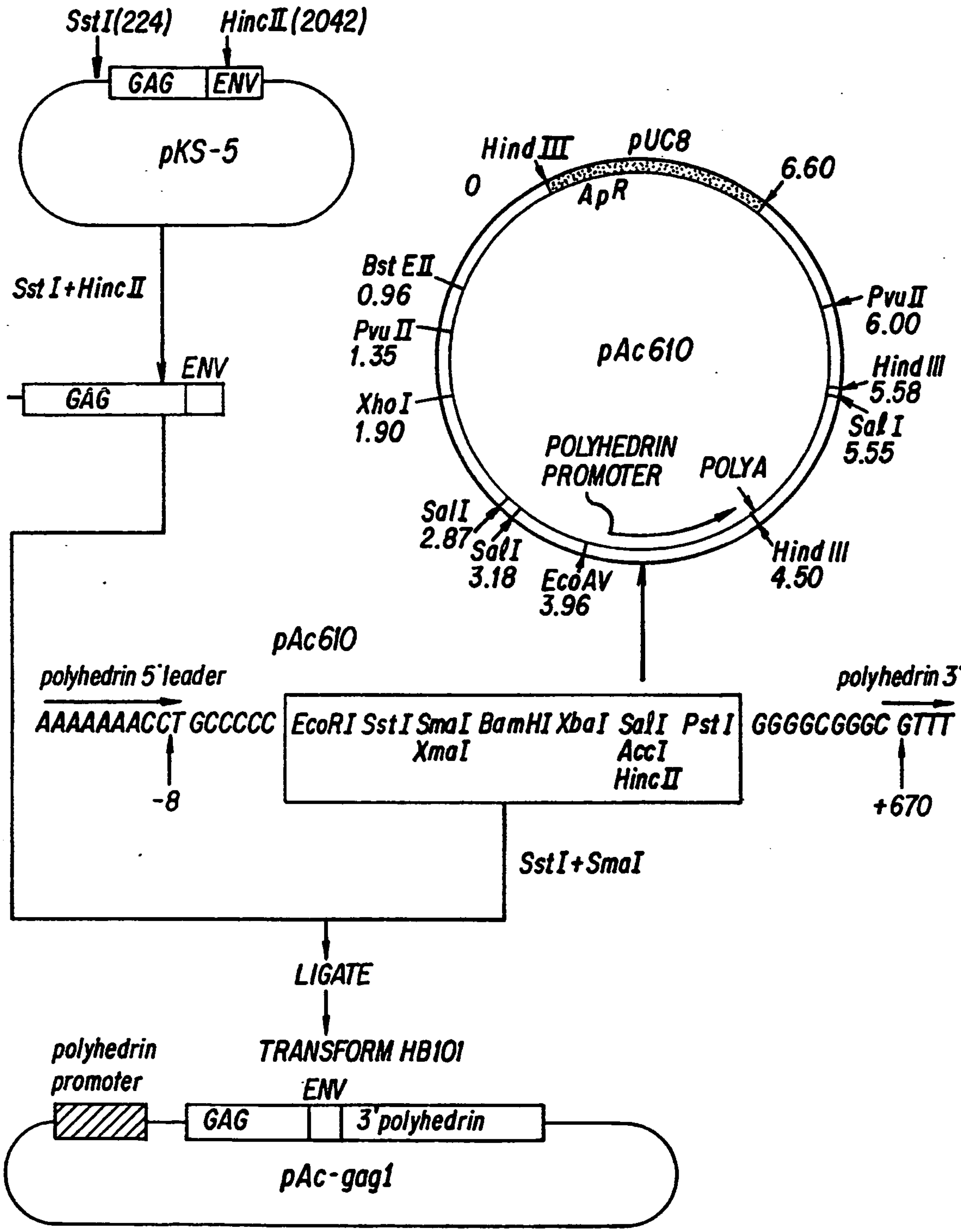
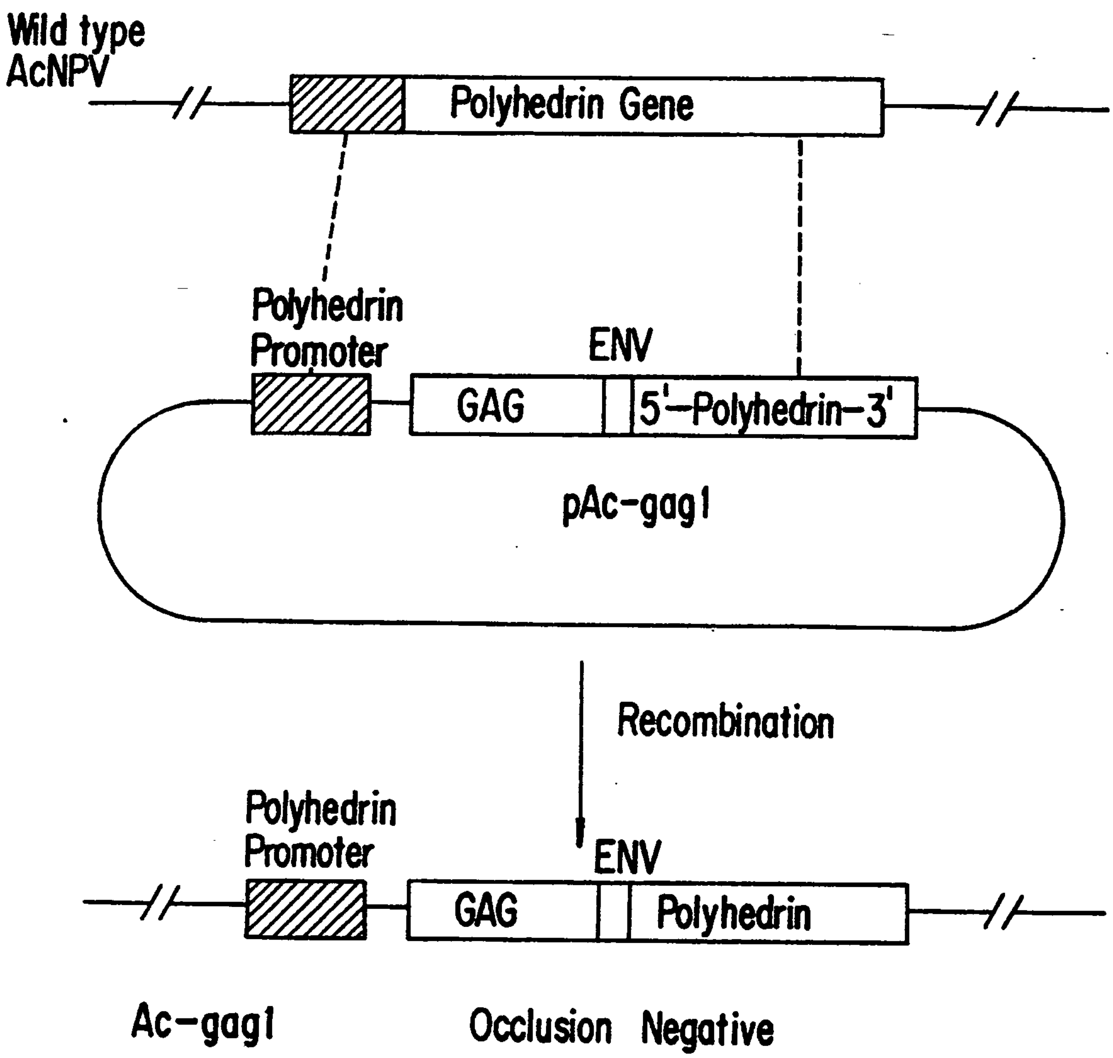

SstI(224)
HincII(2042)
GAG
ENV
pKS-5
SstI+HincII
ENV
GAG
HindIII
pUC8
0
ApR
6.60
BstEII
0.96
PvuII
1.35
XhoI
1.90
pAc610
PvuII
6.00
HindIII
5.58
SalI
5.55
POLYHEDRIN
PROMOTER
POLYA
SalI
2.87
SalI
3.18
EcoAV
3.96
HindIII
4.50
pAc610
polyhedrin 5' leader
AAAAAAACCT GCCCCC
-8
EcoRI SstI SmaI BamHI XbaI SalI PstI
XmaI
AccI
HincII
polyhedrin 3'
GGGGCGGGC GTTT
+670
SstI+SmaI
LIGATE
TRANSFORM HB101
polyhedrin
promoter
ENV
GAG
3'polyhedrin
pAc-gag1

FIG. 15

FIG.16


Wild type
AcNPV
Polyhedrin Gene
Polyhedrin
Promoter
ENV
GAG
5'-Polyhedrin-3'
pAc-gag1
Recombination
Polyhedrin
Promoter
ENV
GAG
Polyhedrin
Ac-gag1
Occlusion Negative

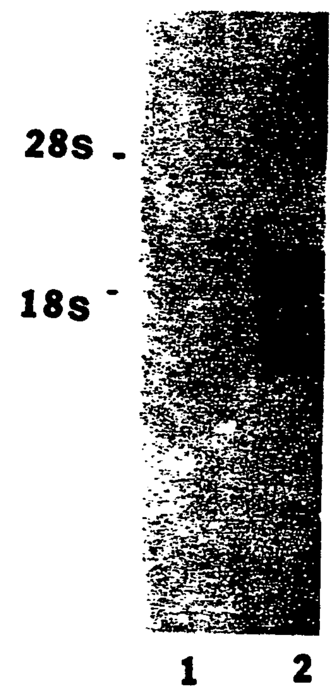


FIG. 17

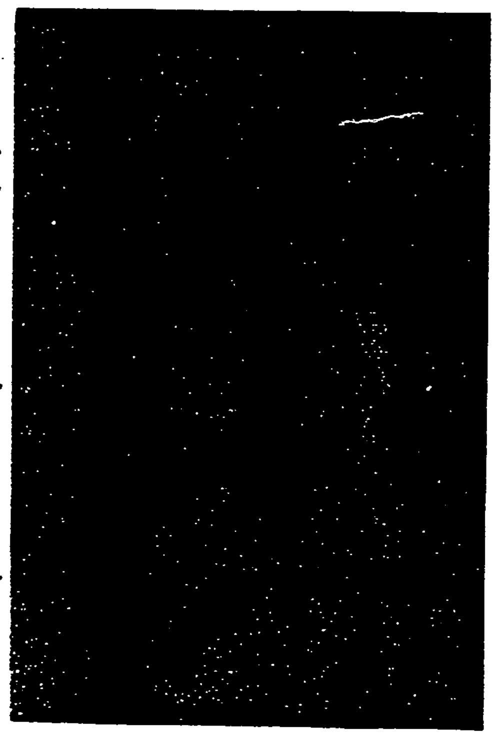


FIG. 18

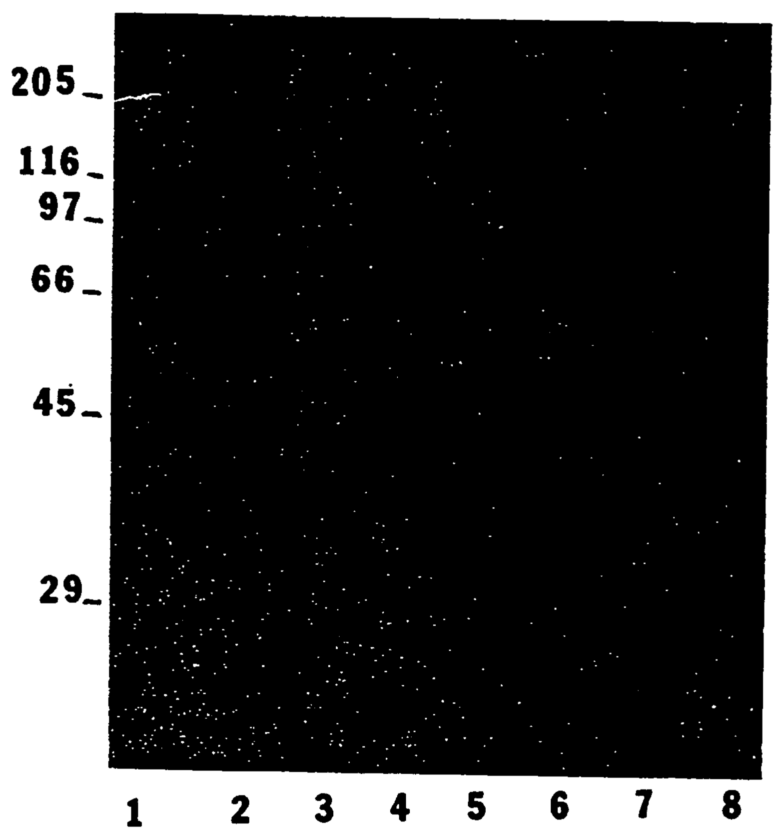


FIG.19

FIG. 20

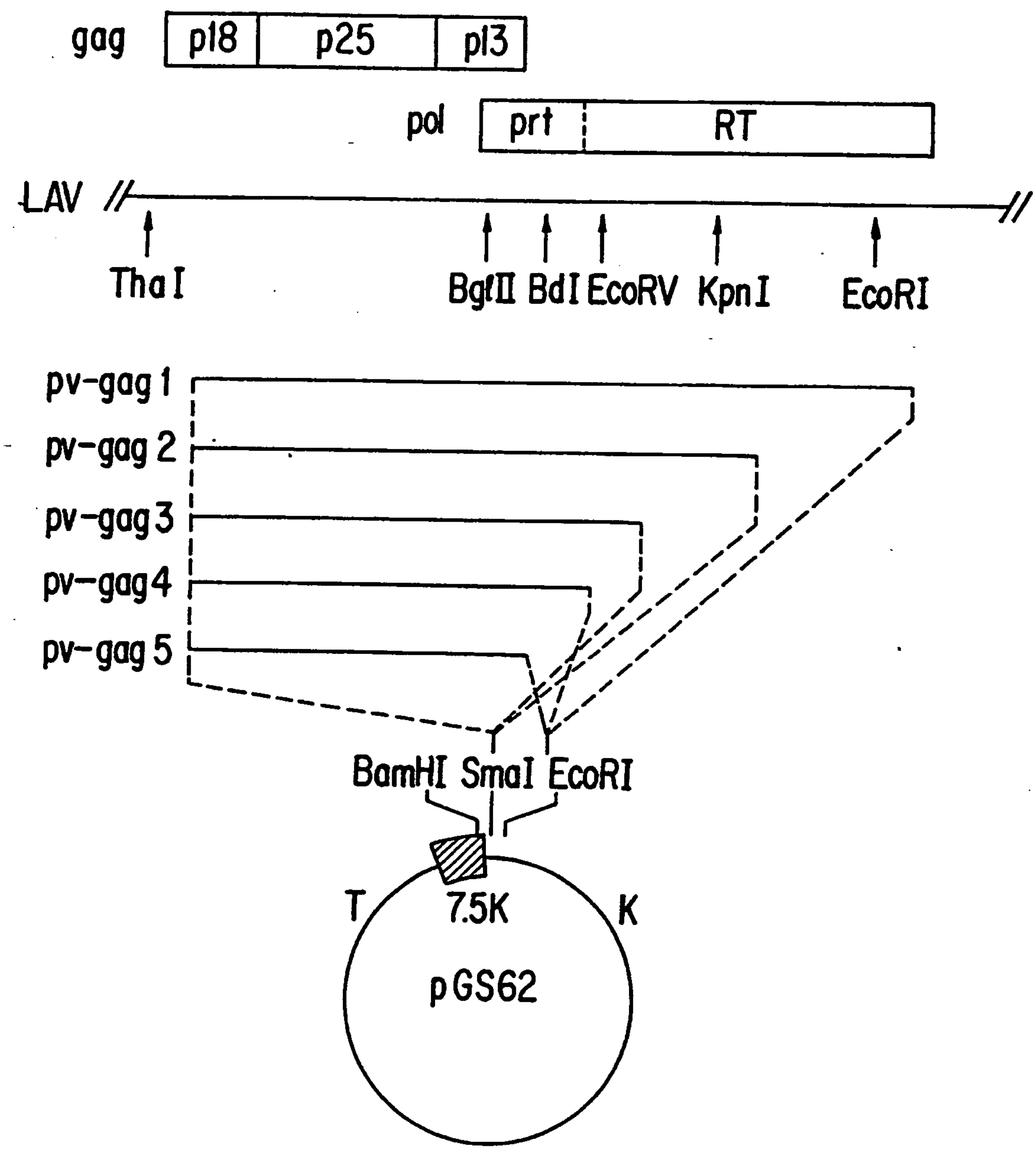

gag
p18
p25
p13
pol
prt
RT
LAV
ThaI
BglII
BdI
EcoRV
KpnI
EcoRI
pv-gag 1
pv-gag 2
pv-gag 3
pv-gag 4
pv-gag 5
BamHI
SmaI
EcoRI
T
7.5K
K
pGS62

FIG. 21

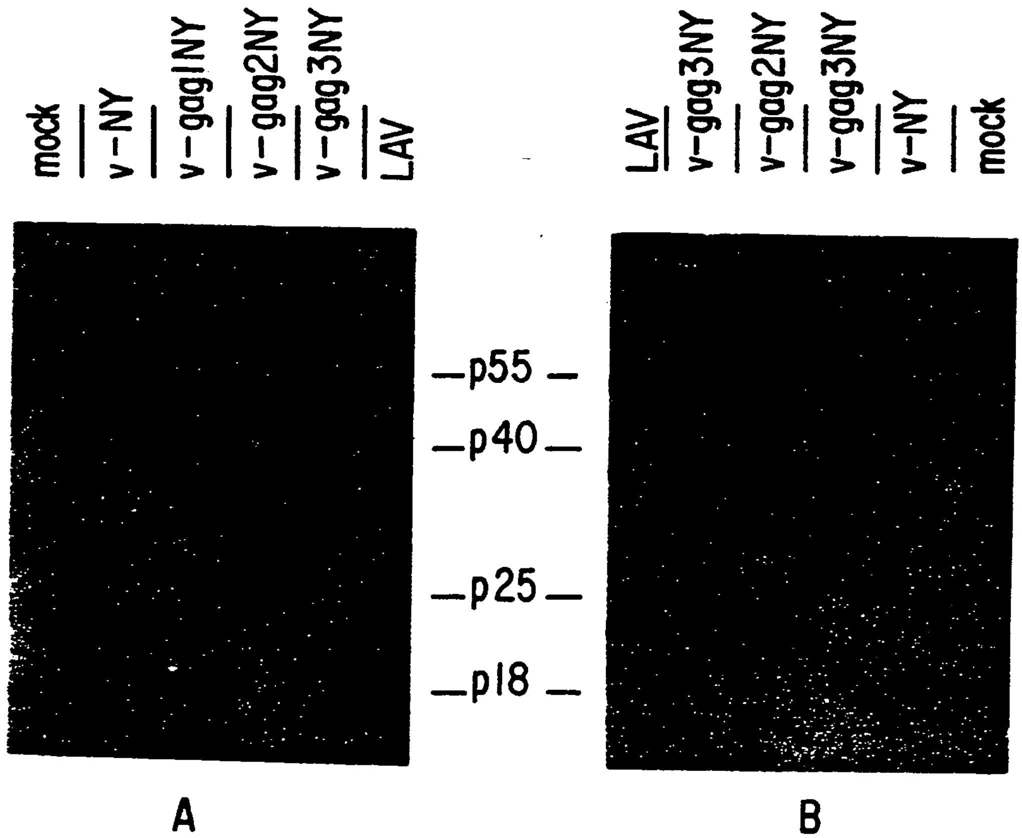
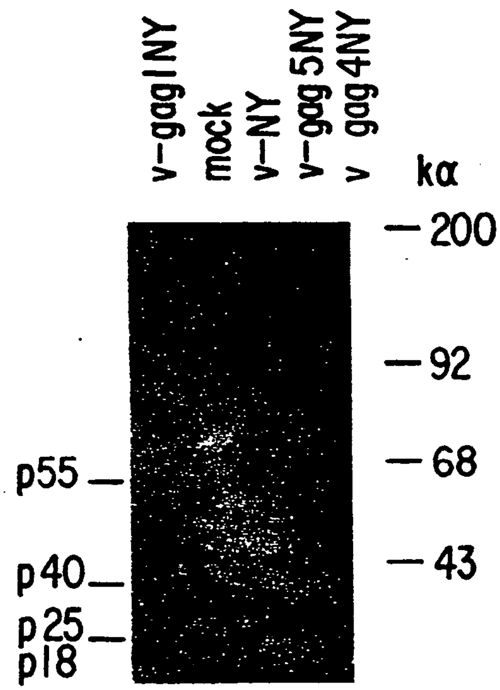
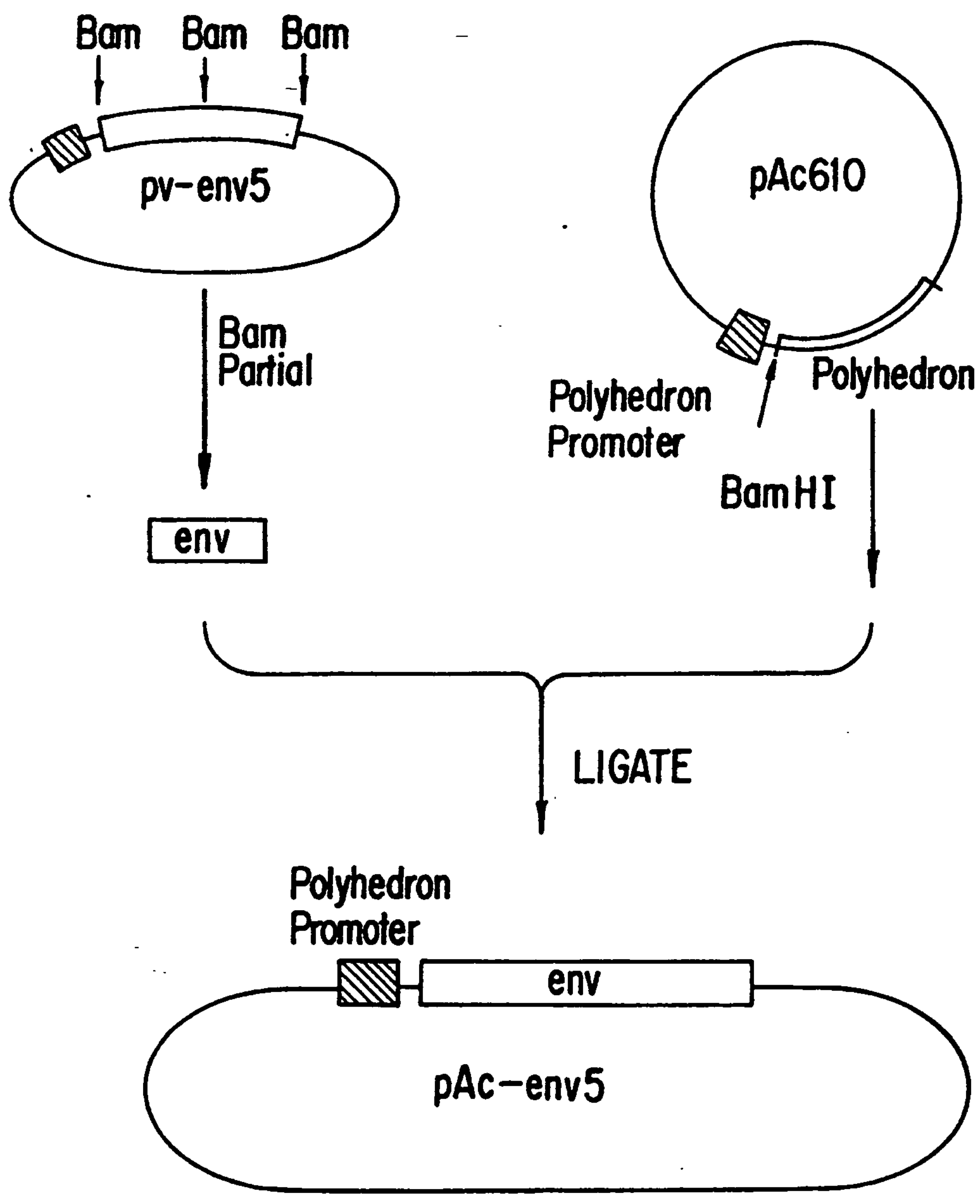

mock
v-NY
v-gag1NY
v-gag2NY
v-gag3NY
LAV
LAV
v-gag3NY
v-gag2NY
v-gag3NY
v-NY
mock
p55
p40
p25
p18
A
B

FIG. 2I


v-gag1NY
mock
v-NY
v-gag5NY
v gag4NY
kα
200
92
68
43
p55
p40
p25
p18

C

FIG. 22


Bam
Bam
Bam
pv-env5
Bam
Partial
env
pAc610
Polyhedron
Promoter
BamHI
Polyhedron
LIGATE
Polyhedron
Promoter
env
pAc-env5

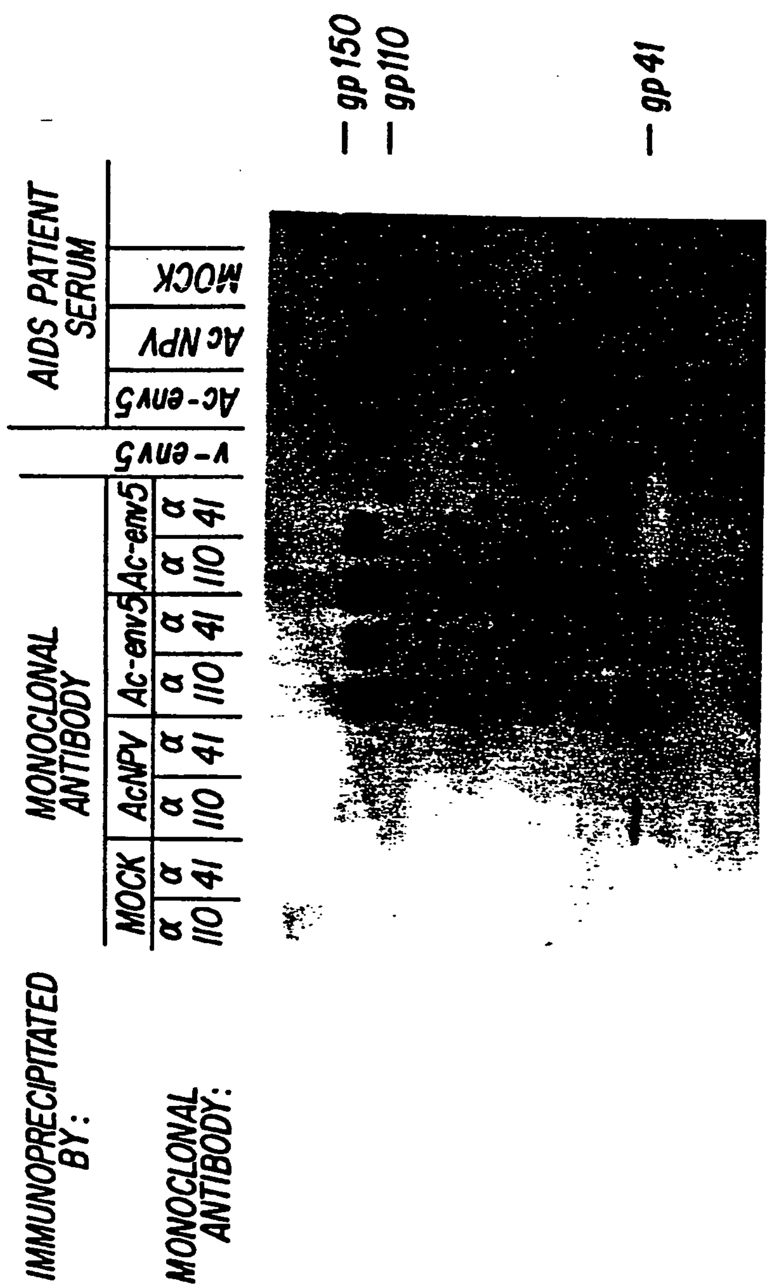

IMMUNOPRECIPITATED BY:
MONOCLONAL ANTIBODY:
MONOCLONAL ANTIBODY
MOCK
AcNPV
Ac-env5
Ac-env5
v-env5
AIDS PATIENT SERUM
Ac-env5
Ac NPV
MOCK
α 110
α 41
α 110
α 41
α 110
α 41
α 110
α 41
— gp150
— gp110
— gp41

FIG. 23

INTERNATIONAL SEARCH REPORT

International Application No PCT/US86/02002

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [3]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC 4 C07K,15/00,15/04;C12N 7/00;C12P,19/34;A61K,39/12,39/21

U.S. 435/172.3 424/89 530/300, 350 514/2

II. FIELDS SEARCHED

Minimum Documentation Searched [4]

Classification System	Classification Symbols
U.S.	435/68,70,91,172.3,253,317;935/29,32,34,39,536/27 530/30, 350,807,424/85,86,89 514/2,12,13,14

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [5]

CHEMICAL ABSTRACTS DATA BASE (CAS) 1967-1986

BIOLOGICAL ABSTRACTS DATA BASE (BIOSIS) 1969-1986

~~KEYWORDS: AIDS, HTLV III; LAV; VACCINE~~

III. DOCUMENTS CONSIDERED TO BE RELEVANT [14]

Category *	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No. [18]
Y	NATURE (London, England), Volume 313, issued 07, February 1985, (MUESING ET AL), "Nucleic Acid structure and expression of human AIDS/lymphadenopathy retrovirus", see entire document.	1-128
Y	CELL (Cambridge, Massachusetts), Volume 41, issued July, 1985, (CROWL ET AL), "HTLV-III env Gene Products Synthesized in E. coli are Recognized by Antibodies Present in the Sera of AIDS Patients", see page 979.	1-128
Y	NATURE (London, England) Volume 312, issued 08, November 1984, (HAHN ET AL), "Molecular cloning of the HTLV-III virus associated with AIDS", see pages 166-167.	1-55 & 101-128

* Special categories of cited documents: [15]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search [2]	Date of Mailing of this International Search Report [2]
09 December 1986	05 JAN 1987
International Searching Authority [1]	**Signature of Authorized Officer [20]**
ISA/US	Ph.D J.D. Stephanie Seidman, Ph.D. J.D.

International Application No. PCT/US/02002

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

Y	PROCEEDINGS NATIONAL ACADEMY OF SCIENCES (Washington D.C. USA), Volume 77,. issued March, 1980, (SNYDER ET AL), "Specificity of human antibodies to oncovirus glycoproteins: Recognition of antigen by natural antibodies directed against carbohydrate structures", see page 1622.	56-100
Y	SCIENCE (Washington, D.C. USA) Volume 224, issued 04, May 1984, (SARNGADHARAN ET AL), "Antibodies Reactive with Human T-Lymphotropic Retroviruses (HTLV-III) in the Serum of Patients with AIRDS", see page 506.	56-100

V.☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [10]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1.☐ Claim numbers ________, because they relate to subject matter [12] not required to be searched by this Authority, namely:

2.☐ Claim numbers ________, because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [13], specifically:

VI.☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [11]

This International Searching Authority found multiple inventions in this international application as follows:

1.☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2.☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3.☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4.☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.